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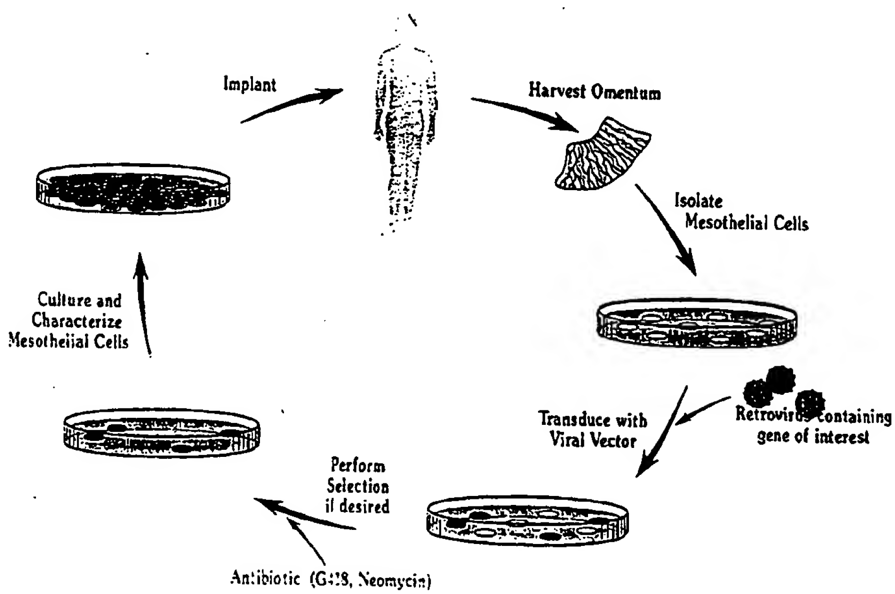

(57) Abstract

Methods and pharmaceutical compositions for modifying the mesothelial cells of a mammalian recipient *in situ* are provided. The methods include forming a mesothelial cell expression system *in vivo* or *ex vivo* and administering the expression system to the mammalian recipient (by way of the body cavities normally lined by mesothelial cells). The mesothelial cell expression system is useful for the localized and systemic delivery of therapeutic agents *in situ*.

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MESOTHELIAL CELL GENE THERAPY

FIELD OF THE INVENTION

This invention relates to gene therapy. More specifically, the present invention relates to mesothelial cell gene therapy in humans and animals.

BACKGROUND OF THE INVENTION

Gene transfer is now widely recognized as a powerful tool for analysis of biological events and disease processes at both the cellular and molecular level (Murray, E.J., ed. Methods in Molecular Biology, Vol. 7, Humana Press Inc., Clifton, N.J., (1991); Kriegler, M., A Laboratory Manual, W.H. Freeman and Co., New York (1990)). More recently, the application of gene therapy for the treatment of human diseases, either inherited (e.g., ADA deficiency) or acquired (e.g., cancer or infectious disease), has received considerable attention (Mulligan, R.C., Science 260:926-932 (1993), Tolstoshev, P., Annu. Rev. Pharmacol. Toxicol. 32:573-596 (1993), Miller, A.D., Nature 357:455-460 (1992), Anderson, W.F., Science 256:808-813 (1992), and references therein). With the advent of improved gene transfer techniques and the identification of an ever expanding library of "defective gene"-related diseases, gene therapy has rapidly evolved from a treatment theory to a practical reality.

Traditionally, gene therapy has been defined as "a procedure in which an exogenous gene is introduced into the cells of a patient in order to correct an inborn genetic error" (Blaese, R.M., Clin. Immunol. Immunopath. 61:S47-S55 (1991)). Although more than 4500 human diseases are currently classified as genetic, (Roemer, K. and Friedmann, T., Eur. J. Biochem. 208:211-225 (1992) and references cited therein), specific mutations in the human genome have been identified for relatively few of these diseases. Until

recently, these rare genetic diseases represented the exclusive targets of gene therapy efforts. Accordingly, most of the N.I.H. approved gene therapy protocols to date have been directed toward the introduction of a functional copy of a defective gene into the somatic cells of an individual having a known inborn genetic error (Miller, A.D., Nature 357:455-460 (1992)). Only recently, have researchers and clinicians begun to appreciate that most human cancers, certain forms of cardiovascular disease, and many degenerative diseases also have important genetic components, and for the purposes of designing novel gene therapies, should be considered "genetic disorders" (Roemer, K. and Friedmann, T., 1992, supra.). Therefore, gene therapy has more recently been broadly defined as "the correction of a disease phenotype through the introduction of new genetic information into the affected organism" (Roemer, K. and Friedmann, T., 1992, supra.).

Two basic approaches to gene therapy have evolved: (1) ex vivo gene therapy and (2) in vivo gene therapy. In ex vivo gene therapy, cells are removed from a subject and cultured in vitro. A functional replacement gene is introduced into the cells (transfection) in vitro, the modified cells are expanded in culture, and then reimplanted in the subject. These genetically modified, reimplanted cells are reported to secrete detectable levels of the transfected gene product in situ (Miller, A.D., Blood 76:271-278 (1990)); Selden, R.F., et al., New Eng. J. Med. 317:1067-1076 (1987)). The development of improved retroviral gene transfer methods (transduction) has greatly facilitated the transfer into and subsequent expression of genetic material by somatic cells (Cepko, C.L., et al., Cell 37:1053-1062 (1984). Accordingly, retrovirus-mediated gene transfer has been used in clinical trials to mark autologous cells and as a way of treating genetic disease (Rosenberg, S.A., et al., New Eng. J. Med. 323:570-578 (1990); Anderson, W.F., Human Gene Therapy 2:99-100 (1991)). Several ex vivo

gene therapy studies in humans have already begun (reviewed in Anderson, W.F., Science 256:808-813 (1992) and Miller A.D., Nature 357:455-460 (1992)).

In in vivo gene therapy, target cells are not removed from the subject. Rather, the transferred gene is introduced into cells of the recipient organism in situ, that is, within the recipient. In vivo gene therapy has been examined in several animal models (reviewed in Felgner, P.L. and Rhodes, G., Nature 349:351-352 (1991)). Several recent publications have reported the feasibility of direct gene transfer in situ into organs and tissues such as muscle (Ferry, N., et al., Proc. Natl. Acad. Sci 88:8377-8781 (1991); Quantin, G., et al., Proc. Natl. Acad. Sci. USA 89:2581-2584 (1992)), hematopoietic stem cells (Clapp, D.W., et al., Blood 78:1132-1139 (1991)), the arterial wall (Nabel, E.G., et al., Science 244:1342-1344 (1989)), the nervous system (Price, J.D., et al., Proc. Natl. Acad. Sci. 84:156-160 (1987)), and lung (Rosenfeld, M.A., et al., Science 252:431-434 (1991)). Direct injection of DNA into skeletal muscle (Wolff, J.A., et al., Science 247:1465-1468 (1990)), heart muscle (Kitsis, R.N., et al., Proc. Natl. Acad. Sci. USA 88:4138-4142 (1991)) and injection of DNA-lipid complexes into the vasculature (Lim, C.S., et al., Circulation 83:2007-2011 (1991); Leclerc, G.D., et al., J. Clin. Invest. 90:936-944 (1992); Chapman, G.D., et al., Circ. Res. 71:27-33 (1992)) also has been reported to yield a detectable expression level of the inserted gene product(s) in vivo.

It was initially assumed that hematopoietic stem cells would be the primary target cell type used for ex vivo human gene therapy (see e.g., Wilson, J.M., et al., Proc. Natl. Acad. Sci. 85:3014-3018 (1988)), in part, because of the large number of genetic diseases associated with differentiated stem cell lineages (Miller, D., Nature 357:455-460 (1992)). However, because of problems inherent to hematopoietic stem cell transfection (e.g., inefficient transgene expression) (Miller, A.D., Blood 76:271-278

(1990)), more recent gene therapy efforts have been aimed at the identification of alternative cell types for transformation. These include: keratinocytes (Morgan, J.R., et al., Science 237:1476-1479 (1987)), fibroblasts (Palmer, T.D., et al., Proc. Natl. Acad. Sci. 88:1330-1334 (1991); Garver Jr., R.I., et al., Science 237:762-764 (1987); International Patent Application PCT/US92/01890, having publication number WO 92/15676), lymphocytes (Reimann, J.K., et al., J. Immunol. Methods 89:93-101 (1986)), myoblasts (Barr, E. and Leiden, J.M., Science 254:1507-1509 (1991); Dai, Y. et al., PNAS 89:10892-10895 (1992); Roman, M., et al., Somatic Cell and Molecular Genetics 18:247-258 (1992)), smooth muscle cells (Lynch, C.M. et al., Proc. Natl. Acad. Sci. USA 89:1138-1142 (1992), and endothelial cells (Nabel, E.G., et al., Science 244:1342-1344 (1989), International Patent Application PCT/US89/05575, having publication number WO 90/06997), the contents of which references and patent/patent applications are incorporated herein by reference.

Despite the wide range of cell types tested, a satisfactory target cell for human gene therapy has not yet been identified. The inadequacies of the above-identified cell types include: (1) inefficient (Williams, et al., 1984; Joyner, et al., 1985) or transient (Mulligan, R.C., Science 260:926-932 (1993)) expression of the inserted gene; (2) potential tumorigenicity of the implanted transduced cell (Selden, et al., 1987; Garver, et al., 1987b); (3) rejection of the implanted genetically modified cell in the absence of harsh immunosuppressive therapy (Selden, et al., 1987); (4) necrosis following subcutaneous injection of cells (Bell, et al., 1983); (5) limited dissemination of the inserted gene product from the site of transduced cell implantation (Morgan, et al., 1987) (see also WO 92/15676); and (6) limitations in the amount of therapeutic agent delivered *in situ*.

The delivery of a therapeutically effective dose of a therapeutic agent in situ depends on both the efficiency of transfection (or transduction), as well as the number of target cells. Thus, despite the potentially high efficiency of transduction using retroviral vectors, many of the above-described cell types are not satisfactory target cells for in vivo gene therapy because of the relatively small numbers of cells available for transduction in situ. Similarly, many of these cell types are not satisfactory for ex vivo gene therapy because of the limited area available in situ for receiving (e.g., by implantation) the genetically modified cells or because of inherent difficulties in accessing a particular anatomical location for implantation of the genetically modified cells.

Endothelial cell-based gene therapy, in particular, is limited by the relatively small area available in situ for receiving genetically modified endothelial cells. Typically, ex vivo gene therapy using endothelial cells requires that a relatively small portion of a blood vessel be sectioned-off to eliminate blood flow through the vessel before removing cells from, or introducing cells to, the blood vessel (Nabel, E.G., et al., Science 244:1342-1344 (1989); Lim, C.S. et al., Circulation 83:2007-2011 (1991); Chapman, G.D. et al., Circulation Res. 71:27-33 (1992)). Consequently, a relatively small number of genetically modified endothelial cells can be implanted into the target vessel. As a result, the delivery of a therapeutically effective dose of therapeutic agent in situ is limited by the total number of implanted endothelial cells.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the instant invention to provide a new method of gene therapy based on the use of genetically engineered mesothelial cells and to the use thereof for delivering a therapeutic agent to a mammalian recipient.

The ideal ex vivo gene therapy method would use a cell that (1) can be easily isolated from the patient, (2) can be modified in vitro to stably express exogenous genetic material (e.g., a therapeutic agent); (3) can be conveniently implanted in the recipient; (4) is nonthrombigenic; and (5) can be implanted into the recipient in large numbers. The ideal in vivo gene therapy method would use a cell that (1) is present in the recipient in large numbers and (2) can be modified in situ to stably express exogenous genetic material (e.g., a therapeutic agent). Preferably, the genetically modified cell would include regulatory elements for controlling the amount of therapeutic agent transcribed and/or expressed, as well as additional elements for directing the therapeutic agent to intracellular, extracellular and/or plasma membrane-associated locations. The preferred genetically modified cells would survive and continue to produce the therapeutic agent in a controlled manner in situ for an amount of time necessary for the therapeutic agent to have a beneficial (i.e., therapeutic) effect, without interfering with the normal function of the tissue in which the cells are located.

The instant invention satisfies these and other objects by providing methods for forming a mesothelial cell expression system, the expression system produced thereby and pharmaceutical compositions containing the same. The mesothelial cell expression system expresses exogenous genetic material (e.g., a heterologous gene encoding a therapeutic agent) and is useful as a vehicle for delivering the gene product to the mammalian recipient in situ. In a preferred embodiment, the mammalian recipient is a human.

In general, the invention relates to genetically engineered mesothelial cells and the use thereof for expressing a therapeutic agent. More particularly, the invention relates to a method for gene therapy which is capable of both localized and systemic delivery of a therapeutically effective dose of a therapeutic agent. Thus,

the invention provides a method for delivering a therapeutically effective amount of a therapeutic agent to any coelomic (pericardial, pleural, and peritoneal) cavity, and subsequently to the systemic circulation by virtue of draining lymphatics, in direct communication with the systemic circulation. Thus, a mesothelial cell expression system is useful for delivering a therapeutic agent throughout the whole patient by, first, the delivery of a therapeutic agent in situ (e.g., to the patient's peritoneal cavity) and then to the systemic circulation of the patient via the interconnecting lymphatic network.

According to one aspect of the invention, a mesothelial cell expression system for expressing a therapeutic agent in a mammalian recipient is provided. The expression system (also referred to herein as a "genetically modified mesothelial cell") comprises a mesothelial cell and an expression vector for expressing the therapeutic agent. Expression vectors of the instant invention include, but are not limited to, viruses, plasmids, and other vehicles for delivering heterologous genetic material to a mesothelial cell. Accordingly, the term "expression vector" as used herein refers to a vehicle for delivering heterologous genetic material to a mesothelial cell.

Preferably, the expression vector further includes a promoter for controlling transcription of the heterologous gene. More preferably, the promoter is an inducible promoter (described below). The expression system is suitable for administration to the mammalian recipient. In a preferred embodiment, the expression system comprises a plurality of nonimmortalized mesothelial cells, each cell containing at least one recombinant gene encoding at least one therapeutic agent.

The mesothelial cell expression system can be formed ex vivo or in vivo. To form the expression system ex vivo, one or more isolated mesothelial cells are transduced with a virus or transfected with a nucleic acid or plasmid in

vitro. Preferably the transduced or transfected mesothelial cells are thereafter expanded in culture and thereafter administered to the mammalian recipient for delivery of the therapeutic agent in situ. In a preferred embodiment, the mesothelial cell comprises an autologous cell, i.e., the cell is isolated from the mammalian recipient. The genetically modified cell(s) are administered to the recipient by, for example, implanting the cell(s) or a graft (or capsule) including a plurality of the cells into a mesothelial cell-compatible site of the recipient. Representative mesothelial cell-compatible sites include, for example, the peritoneal, pleural and pericardial cavities. Preferably, the mesothelial cell-compatible site is denuded, i.e., a section of mesothelial cells is removed, to expose the underlying basement membrane, prior to implanting the genetically modified cells.

According to yet another aspect of the invention, a method for genetically modifying the mesothelial system of a mammalian recipient (preferably a human) in vivo is provided. The method comprises introducing an expression vector for expressing a heterologous gene product into a mesothelial cell of the patient in situ. To form the expression system in vivo, an expression vector for expressing the therapeutic agent is introduced in vivo into a coelomic cavity (e.g., peritoneal cavity) of the mammalian recipient by, for example, intraperitoneal injection.

Preferably, the expression vector for expressing the heterologous gene includes an inducible promoter for controlling transcription of the heterologous gene product. Accordingly, delivery of the therapeutic agent in situ is controlled by exposing the cell in situ to conditions which induce transcription of the heterologous gene.

In the preferred embodiments, the mammalian recipient has a condition that is amenable to gene replacement therapy. As used herein, "gene replacement therapy" refers to administration to the recipient of exogenous genetic

material encoding a therapeutic agent and subsequent expression of the administered genetic material _in situ_. Thus, the phrase "condition amenable to gene replacement therapy" embraces conditions such as genetic diseases (i.e., a disease condition that is attributable to one or more gene defects), acquired pathologies (i.e., a pathological condition which is not attributable to an inborn defect), cancers and prophylactic processes (i.e., prevention of a disease or of an undesired medical condition). Accordingly, as used herein, the term "therapeutic agent" refers to any agent or material which has a beneficial effect on the mammalian recipient. Thus, "therapeutic agent" embraces both therapeutic and prophylactic molecules having nucleic acid (e.g., antisense RNA) and/or protein components.

According to one preferred embodiment, the mammalian recipient has a genetic disease and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the disease. In yet another embodiment, the mammalian recipient has an acquired pathology and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the pathology. According to another embodiment, the patient has a cancer and the exogenous genetic material comprises a heterologous gene encoding an anti-neoplastic agent. In yet another embodiment the patient has an undesired medical condition and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the condition. Thus, exemplary therapeutic agents (with the conditions they treat appearing in the parentheses) include Factor VIII (hemophilia A) and Factor IX (hemophilia B), adenosine deaminase (severe combined immunodeficiency disease), erythropoietin (anemia), and tumor necrosis factor (cancer). Lists of these and other therapeutic agents are provided in Tables 1-3. Exemplary therapeutic agents that are prophylactic agents for treating a prophylactic process (with their use appearing in parentheses) include thyroxine (for

treating hypothyroidism), estrogen/progesterone (as contraceptive agents), albumin (as an oncotic agent in peritoneal dialysis).

According to yet another embodiment, a pharmaceutical composition is disclosed. The pharmaceutical composition comprises a plurality of the above-described genetically modified mesothelial cells and a pharmaceutically acceptable carrier. Preferably, the pharmaceutical composition is for treating a condition amenable to gene replacement therapy and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the condition. More preferably, the pharmaceutical composition contains an amount of genetically modified cells sufficient to deliver a therapeutically effective dose of the therapeutic agent to the patient. Exemplary conditions amenable to gene replacement therapy are described below.

According to another aspect of the invention, a method for forming the above-described pharmaceutical composition is provided. The method includes introducing an expression vector for expressing a heterologous gene product into a mesothelial cell to form a genetically modified mesothelial cell and placing the genetically modified cell in a pharmaceutically acceptable carrier.

According to still another aspect of the invention, a mesothelial cell graft is disclosed. The graft comprises a plurality of genetically modified mesothelial cells attached to a support which is suitable for implantation into the mammalian recipient. The support may be formed of a natural or synthetic material. According to one embodiment, the mesothelial cell graft comprises a patch of peritoneum including a plurality of mesothelial cells, which cells contain a recombinant gene. In a preferred embodiment, the cells are genetically modified ex vivo following excision of the patch from the mammalian recipient.

According to still another aspect of the invention, an encapsulated mesothelial cell expression system is disclosed. The encapsulated expression system comprises a plurality of genetically modified mesothelial cells contained within a capsule which is suitable for implantation into the mammalian recipient. The capsule may be formed of a natural or synthetic material. Preferably, the capsule containing the plurality of genetically modified mesothelial cells is implanted in the peritoneal cavity.

These and other aspects of the invention as well as various advantages and utilities will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a growth curve of rat primary mesothelial cells *in vitro*.

Figure 2 is a schematic diagram of a method of human mesothelial cell gene therapy.

Figure 3 shows the *in vitro* production of hgh by stably transfected rat mesothelial cells (clone meso gh 2-3).

Figure 4 shows hgh production *in vivo* by transfected rat mesothelial cells reseeded in the peritoneal cavities of Fisher rats compared to hgh levels in normal control rats. A. Time course of plasma hgh levels over hours. B. Time course of plasma hgh levels over days.

Figure 5 shows the presence of an inhibitor in serum from rats implanted with hgh-secreting mesothelial cells. A. Time course of appearance of inhibitory activity. B. Inhibitory titer determinations.

Figure 6 shows hgh production *in vivo* in immunosuppressed animals (dexamethasone-treated rats).

Figure 7 shows hgh production *in vitro* by stably transfected uncloned human meosthelial cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The instant invention provides a mesothelial cell expression system for expressing exogenous genetic material in a mammalian recipient. The expression system, also referred to as a "genetically modified mesothelial cell", comprises a mesothelial cell and an expression vector for expressing the exogenous genetic material. The genetically modified mesothelial cells are suitable for administration to a mammalian recipient, where they replace the endogenous mesothelial cells of the recipient. Thus, the preferred genetically modified cells are nonimmortalized and are nontumorigenic.

The mesothelial cells of the instant invention are parietal, visceral surface or free-floating mesothelial cells that are present in, or derived from, a simple squamous epithelium that forms the limiting serosal membranes that line the coelomic cavities (i.e., pleural, pericardial, and peritoneal cavities) (reviewed in (Whitaker, D., et al., CRC Critical Reviews in Toxicology 10:81-144 (1982); Dobbie, J.W., Am. J. Kid. Dis. 15:97-109 (1990); Gotloib, L. and Shostak, A., Peritoneal Dialysis, K.D. Nolph, Editor, Nijhoff, Amsterdam:. 67-95 (1989)). Mesothelial cells function by providing a frictionless surface which facilitates movement within the pleural, pericardial, and peritoneal cavities (Whitaker, D., et al. supra). In addition, the mesothelial cells possess a secretary function (Di Paolo, N., Perit. Dial. Int. 9:151-153 (1989); Dobbie, J.W., Perit. Dial. Int. 8:3-6 (1988)) and by virtue of their anatomical location in the peritoneal cavity, are amenable to removal and subsequent reimplantation (Di Paolo, N., et al., Int. J. Art. Org. 12:485-501 (1989); Di Paolo, N., et al., Clinical Nephrol. 34:179-1848 (1990); Di Paolo, N., et al., Nephron 57:323-331 (1991)).

According to one embodiment, the mesothelial cells are transformed or otherwise genetically modified *ex vivo*. The mesothelial cells are isolated from a mammal (preferably a

human), transformed (i.e., transduced or transfected in vitro with a vector for expressing a heterologous (e.g., recombinant) gene encoding the therapeutic agent, and then administered to a mammalian recipient for delivery of the therapeutic agent in situ. Preferably, the mammalian recipient is a human and the mesothelial cells to be modified are autologous cells, i.e., the cells are isolated from the mammalian recipient. The isolation and culture of mesothelial cells in vitro has been reported (see e.g., Stylianou, E., et al., Kidney Intl. 37:1563-1570 (1990); Pronk, A. et al. In Vitro Cell. Dev. Biol. 29A:127-134 (1993)), as has the implantation of autologous peritoneal mesothelial cells in peritoneal dialysis patients (see e.g., Di Paolo, N., et al., Nephron. 57:323-331 (1991) and references cited therein).

According to another embodiment, the mesothelial cells are transformed or otherwise genetically modified in vivo. The mesothelial cells from the mammalian recipient (preferably a human), are transformed (i.e., transduced or transfected) in vivo with a vector containing exogenous genetic material for expressing a heterologous (e.g., recombinant) gene encoding a therapeutic agent and the therapeutic agent is delivered in situ.

As used herein, "exogenous genetic material" refers to a nucleic acid or an oligonucleotide, either natural or synthetic, that is not naturally found in mesothelial cells; or if it is naturally found in the cells, it is not transcribed or expressed at biologically significant levels by mesothelial cells. Thus, "exogenous genetic material" includes, for example, a non-naturally occurring nucleic acid that can be transcribed into anti-sense RNA, as well as a "heterologous gene" (i.e., a gene encoding a protein which is not expressed or is expressed at biologically insignificant levels in a naturally-occurring mesothelial cell). To illustrate, a synthetic or natural gene encoding human erythropoietin (EPO) would be considered "exogenous genetic

material" with respect to human peritoneal mesothelial cells since the latter cells do not naturally express EPO; similarly, a human interleukin-1 gene inserted into a peritoneal mesothelial cell would also be an exogenous gene to that cell since peritoneal mesothelial cells do not naturally express interleukin-1 at biologically significant levels. Still another example of "exogenous genetic material" is the introduction of only part of a gene to create a recombinant gene, such as combining an inducible promoter with an endogenous coding sequence via homologous recombination.

In the preferred embodiments, the mammalian recipient has a condition that is amenable to gene replacement therapy. As used herein, "gene replacement therapy" refers to administration to the recipient of exogenous genetic material encoding a therapeutic agent and subsequent expression of the administered genetic material in situ. Thus, the phrase "condition amenable to gene replacement therapy" embraces conditions such as genetic diseases (i.e., a disease condition that is attributable to one or more gene defects), acquired pathologies (i.e., a pathological condition which is not attributable to an inborn defect), cancers and prophylactic processes (i.e., prevention of a disease or of an undesired medical condition). Accordingly, as used herein, the term "therapeutic agent" refers to any agent or material which has a beneficial effect on the mammalian recipient. Thus, "therapeutic agent" embraces both therapeutic and prophylactic molecules having nucleic acid (e.g., antisense RNA) and/or protein components.

A number of diseases caused by single-gene defects have been identified (Roemer, K. and Friedmann, T., Eur J. Biochem. 208:211-225 (1992); Miller, A.D., Nature 357:455-460 (1992); Larrick, J.W. and Burck, K.L. Gene Therapy. Application of Molecular Biology, Elsevier, New York, (1991) and references contained therein). Examples of these diseases, and the therapeutic agents for treating the exemplary diseases, are provided in Table 1.

Table 1

Therapeutic Agents for Treating Diseases Involving Single-Gene Defects*

Disease	Therapeutic Agent
Immunodeficiency	Adenosine deaminase
	Purine nucleoside phosphorylase
Hypercholesterolaemia	LDL receptor
Haemophilia A	Factor VIII
Haemophilia B	Factor IX
Gaucher's disease	Glucocerebrosidase
Mucopolysaccharidosis	ß-glucuronidase
Emphysema	α_1-antitrypsin
Cystic fibrosis	Cystic fibrosis trans-membrane regulator
Phenylketonuria	Phenylalanine hydroxylase
Hyperammonaemia	Ornithine transcarbamylase
Citrullinaemia	Arginosuccinate synthetase
Muscular dystrophy	Dystrophin
Thalassaemia	ß-globin
Sickle cell anaemia	α-globin
Leukocyte adhesion deficiency	CD-18
von Willebrand's disease	von Willebrand Factor

*see Roemer, K. and Friedmann, T., Eur J. Biochem. 208:211-225 (1992) and Miller, A.D., 1992, Nature 357:455-4-60 and references contained therein.

As used herein, "acquired pathology" refers to a disease or syndrome manifested by an abnormal physiological, biochemical, cellular, structural, or molecular biological state. Exemplary acquired pathologies, and the therapeutic agents for treating the exemplary pathologies, are provided in Table 2.

The condition amenable to gene replacement therapy alternatively can be a genetic disorder or an acquired pathology that is manifested by abnormal cell proliferation, e.g., cancers arising in or metastasizing to the coelomic cavities. According to this embodiment, the instant invention is useful for delivering a therapeutic agent having anti-neoplastic activity (i.e., the ability to prevent or inhibit the development, maturation or spread of abnormally growing cells), to tumors arising in or metastasizing to the coelomic cavities, (e.g., ovarian carcinoma, mesothelioma, colon carcinoma). Therapeutic agents for treating these and other cancers include, for example, the anti-neoplastic agents provided in Table 3.

Delivery of a therapeutic agent by a genetically modified mesothelial cell is not limited to delivery to the coelomic cavity in which the genetically modified mesothelial cells reside. By virtue of the anatomical location of the coelomic cavities, a therapeutic agent secreted by a genetically modified mesothelial cell within a coelomic cavity could reach the lymphatic network draining that coelomic cavity. Accordingly, the genetically modified mesothelial cells of the invention are useful for delivering a therapeutic agent, such as an anti-neoplastic agent, to the coelomic cavities (e.g., peritoneal, pleural, pericardial), to the lymphatic network into which these cavities drain and to the vascular system via the interconnecting lymphatic network. Therefore a therapeutic agent, such as an anti-neoplastic agent, delivered by genetically modified

Table 2

Therapeutic Agents for Acquired Pathologies

Associated with Peritoneal Dialysis	Therapeutic Agent
Anemia	Erythropoietin
Peritoneal sclerosis	Fibrinolytic agents (e.g., tissue plasminogen activator (t-PA), or single chain urokinase plasminogen activator (scu-PA)
Peritonitis	Anti-oxidants (e.g., Superoxide Dismutase, Catalase)
Uremia	Urease

Other Conditions	Therapeutic Agent
Septic Shock	Anti-thrombotic agents (e.g., elastase-resistant form of thrombomodulin (TM))
Diabetes mellitus	Insulin
Pituitary Dwarfism	Human growth hormone
Thrombosis	Hirudin, secreted form of TM
Post-Surgical Adhesions	Anti-thrombotic agents (e.g., thrombomodulin, hirudin) Fibrinolytic agents (e.g., TPA, scu-PA) Surfactants
AIDS	CD-4

Table 3

Therapeutic Agents for Treating Cancers*

Defective Gene	Therapeutic Agent
Oncogenes	corresponding normal genes, oncogene antisense RNA
Mutated Tumor-Suppressor genes	Normal Tumor-Suppressor (e.g., p53)
Unidentified defect	cytokines, the interferons, tumor necrosis factor, the interleukins

*see Roemer, K. and Friedmann, T., 1992, supra., and references contained therein.

mesothelial cells, would reach cancer cells within the coelomic cavities, within the draining lymphatics and at distant metastatic sites.

Alternatively, the condition amenable to gene replacement therapy is a prophylactic process, i.e., a process for preventing disease or an undesired medical condition. Thus, the instant invention embraces a mesothelial cell expression system for delivering a therapeutic agent that has a prophylactic function (i.e., a prophylactic agent) to the mammalian recipient. Such therapeutic agents (with the disease or undesired medical condition they prevent appearing in parentheses) include: estrogen/ progesterone (pregnancy); thyroxine (hypothyroidism); and agents which stimulate, e.g., gamma-interferon, or supplement, e.g., antibodies, the immune system response (diseases associated with deficiencies of the immune system).

In summary, the term "therapeutic agent" includes, but is not limited to, the agents listed in Tables 1-3, as well as their functional equivalents. As used herein, the term "functional equivalent" refers to a molecule (e.g., a peptide or protein) that has the same or an improved beneficial effect on the mammalian recipient as the therapeutic agent of which is it deemed a functional equivalent. As will be appreciated by one of ordinary skill in the art, a functionally equivalent protein can be produced by recombinant techniques, e.g., by expressing a "functionally equivalent DNA". As used herein, the term "functionally equivalent DNA" refers to a non-naturally occurring DNA which encodes a therapeutic agent. For example, many, if not all, of the agents disclosed in Tables 1-3 have known amino acid sequences, which are encoded by naturally occurring nucleic acids. However, due to the degeneracy of the genetic code, more than one nucleic acid can encode the same therapeutic agent. Accordingly, the instant invention embraces

therapeutic agents encoded by naturally-occurring DNAS, as well as by nonnaturally-occurring DNAs which encode the same protein as encoded by the naturally-occurring DNA.

The above-disclosed therapeutic agents and conditions amenable to gene replacement therapy are merely illustrative and are not intended to limit the scope of the instant invention. The selection of a suitable therapeutic agent for treating a known condition is deemed to be within the scope of one of ordinary skill of the art without undue experimentation.

Methods for Introducing Genetic Material into Mesothelial Cells

The exogenous genetic material (e.g., a cDNA encoding one or more therapeutic proteins) is introduced into the mesothelial cell *ex vivo* or *in vivo* by genetic transfer methods, such as transfection or transduction, to provide a genetically modified mesothelial cell. Various expression vectors (i.e., vehicles for facilitating delivery of exogenous genetic material into a target cell) are known to one of ordinary skill in the art.

As used herein, "transfection of mesothelial cells" refers to the acquisition by a mesothelial cell of new genetic material by incorporation of added DNA. Thus, transfection refers to the insertion of nucleic acid into a mesothelial cell using physical or chemical methods. Several transfection techniques are known to those of ordinary skill in the art including: calcium phosphate DNA co-precipitation (Methods in Molecular Biology, Vol. 7, Gene Transfer and Expression Protocols, Ed. E.J. Murray, Humana Press (1991)); DEAE-dextran (supra); electroporation (supra); cationic liposome-mediated transfection (supra); and tungsten particle-faciliated microparticle bombardment (Johnston, S.A., Nature 346:776-777 (1990)). Strontium phosphate DNA co-precipitation (Brash D.E. et al. Molec. Cell. Biol. 7:2031-2034 (1987) is the preferred transfection method.

In contrast, "transduction of mesothelial cells" refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. A RNA virus (i.e., a retrovirus) for transferring a nucleic acid into a cell is referred to herein as a transducing chimeric retrovirus. Exogenous genetic material contained within the retrovirus is incorporated into the genome of the transduced mesothelial cell. A mesothelial cell that has been transduced with a chimeric DNA virus (e.g., an adenovirus carrying a cDNA encoding a therapeutic agent), will not have the exogenous genetic material incorporated into its genome but will be capable of expressing the exogenous genetic material that is retained extrachromosomally within the cell.

U.S. Patent No. 4,885,238, issued December 5, 1989 to Reddel et al., the contents of which are incorporated herein by reference, discloses immortalized human bronchial epithelial and human mesothelial cell lines. The immortalized cells were prepared by culturing normal human mesothelial (NHM) cells from pleural effusions or ascites fluids as described by Lechner et al, (Proc. Natl. Acad. Sci. U.S.A. 82:3884-3888, 1985) and thereafter transducing the cultured cells with SV40 virus or with an adenovirus-12 SV40 chimeric virus, or transfecting the cultured cells with a recombinant plasmid containing the Rous sarcoma virus long terminal repeat and the ori-SV40 early region by strontium phosphate co-precipitation (Brash et al. Molec. Cell. Biol. 7: 2031-20341987). The term "immortalized cell" as used in the Reddel et al., patent, means a cell which grows continually without senescence when cultured in vitro in a suitable growth medium. Accordingly, the transformed mesothelial cells of Reddel et al., are immortalized and are not suitable for direct implantation into a mammalian recipient. Thus, the Reddel et al. cells are limited to a variety of in vitro applications. Immortalized mesothelial cell lines are also disclosed in Yang Ke, et al., Amer. J. Pathology 134:979-991 (1989), on which reference H. Reddel is

a co-author, and Rheinwald, J., et al., Neoplastic Transformation in Human Cell Culture, Eds.: J.S. Rhim and A. Dritschilo, (1991), The Humana Press Inc., Totowa, N.J.).

Typically, the exogenous genetic material includes the heterologous gene (usually in the form of a cDNA comprising the exons coding for the therapeutic protein) together with a promoter to control transcription of the new gene. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (i.e., enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any nontranslated DNA sequence which works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. Preferably, the exogenous genetic material is introduced into the mesothelial cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A preferred retroviral expression vector includes an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR) (Scharfmann et al., Proc. Natl. Acad. Sci. USA 88:4626-4630 (1991)), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter (Lai et al., Proc. Natl. Acad. Sci. USA 86: 10006-10010 (1989)), and other constitutive promoters known to those of skill in the art.

In addition, many viral promoters function constitutively in eucaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRS) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (e.g., transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified mesothelial cell. If the gene encoding the therapeutic agent is under the control of an inducible promoter, delivery of the therapeutic agent _in situ_ is triggered by exposing the genetically modified cell _in situ_ to conditions for permitting transcription of the therapeutic agent, e.g., by intraperitoneal injection of specific inducers of the inducible promoters which control transcription of the agent. For example, _in situ_ expression by genetically modified mesothelial cells of a therapeutic agent encoded by a gene under the control of the metallothionein promoter, is enhanced by contacting the genetically modified cells with a solution containing the appropriate (i.e., inducing) metal ions _in situ_.

Accordingly, the amount of therapeutic agent that is delivered in situ is regulated by controlling such factors as: (1) the nature of the promoter used to direct transcription of the inserted gene, (i.e., whether the promoter is constitutive or inducible, strong or weak); (2) the number of copies of the exogenous gene that are inserted into the mesothelial cell; (3) the number of transduced/transfected mesothelial cells that are administered (e.g., implanted) to the patient; (4) the size of the implant (e.g., graft or encapsulated expression system); (5) the number of implants; (6) the length of time the transduced/transfected cells or implants are left in place; and (7) the production rate of the therapeutic agent by the genetically modified mesothelial cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector preferably includes a selection gene, for example, a neomycin resistance gene, for facilitating selection of mesothelial cells that have been transfected or transduced with the expression vector. Alternatively, the mesothelial cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence (described below) is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

The therapeutic agent can be targeted for delivery to an extracellular, intracellular or membrane location. If it is desirable for the gene product to be secreted from the

mesothelial cells (e.g., to deliver the therapeutic agent to the lymphatic and vascular systems), the expression vector is designed to include an appropriate secretion "signal" sequence for secreting the therapeutic gene product from the cell to the extracellular milieu. If it is desirable for the gene product to be retained within the mesothelial cell, this secretion signal sequence is omitted. In a similar manner, the expression vector can be constructed to include "retention" signal sequences for anchoring the therapeutic agent within the mesothelial cell plasma membrane. For example, all membrane proteins have hydrophobic transmembrane regions which stop translocation of the protein in the membrane and do not allow the protein to be secreted. The construction of an expression vector including signal sequences for targeting a gene product to a particular location is deemed to be within the scope of one of ordinary skill in the art without the need for undue experimentation.

The following discussion is directed to various utilities of the instant invention. For example, the instant invention has utility as an expression system suitable for detoxifying intra- and/or extracellular toxins <u>in situ</u>. By attaching or omitting the appropriate signal sequence to a gene encoding a therapeutic agent capable of detoxifying a toxin, the therapeutic agent can be targeted for delivery to the extracellular milieu, to the mesothelial cell plasma membrane or to an intracellular location. In a preferred embodiment, the exogenous genetic material containing a gene encoding an intracellular detoxifying therapeutic agent, further includes sequences encoding surface receptors for facilitating transport of extracellular toxins into the cell where they can be detoxified intracellularly by the therapeutic agent. Alternatively, the mesothelial cells can be genetically modified to express the detoxifying therapeutic agent anchored within the mesothelial cell plasma membrane such that the active portion extends into the extracellular milieu. The active portion of the

membrane-bound therapeutic agent detoxifies toxins which are present in the extracellular milieu. The above-described embodiment can be useful in the treatment of end-stage renal disease patients, who accumulate toxic substances (e.g., beta-2 microglobulin) in their blood. Implantation of genetically modified mesothelial cells expressing a therapeutic agent for detoxifying or removing accumulated toxins could decrease the morbidity associated with these toxins. Other pathologies, and their corresponding detoxifying therapeutic agent(s), for which the above described embodiment would apply include, but are not limited to: hypercholesterolaemia (phospholipase A_2 or LDL receptor); phenylketonuria (phenylalanine hydroxylase); hyperammonaemia (ornithine transcarbamylase); citrullinaemia (arginosucccinate synthetase); and hyperbilirubinemia (bilirubin decarboxylase) (Robbins, S.L., Cotran, R.S. and Kumar, V., Pathologic Basis of Disease, 3rd edition, W.B. Saunders Co., Philadelphia, (1984)).

In addition to the above-described therapeutic agents, some of which are targeted for intracellular retention, the instant invention also embraces agents intended for delivery to the extracellular milieu and/or agents intended to be anchored in the mesothelial cell plasma membrane. For example, expression of an anti-thrombotic agent, such as thrombomodulin, on the surface of a plurality of mesothelial cells implanted onto the peritoneal wall can prevent clotting as well as reduce the incidence of post-surgical adhesions by preventing the deposition of fibrin. Other examples of therapeutic agents suitable for expression on the surface of a genetically modified mesothelial cell include, but are not limited to: LDL receptor; single chain urokinase plasminogen activator (scu-PA); and tissue type plasminogen activator (t-PA).

The selection and optimization of a particular expression vector for expressing a specific gene product in an isolated mesothelial cell is accomplished by obtaining the

gene, preferably with one or more appropriate control regions (e.g., promoter, insertion sequence); preparing a vector construct comprising the vector into which is inserted the gene; transfecting or transducing cultured mesothelial cells in vitro with the vector construct; and determining whether the gene product is present in the cultured cells.

In a preferred embodiment, vectors for mesothelial cell gene therapy are viruses, more preferably replication-deficient viruses (described in detail below). Exemplary viral vectors are derived from: Harvey Sarcoma virus; ROUS Sarcoma virus, (MPSV); Moloney murine leukemia virus and DNA viruses (e.g., adenovirus) (Temin, H., "Retrovirus vectors for gene transfer", in Gene Transfer Kucherlapati R, Ed., pp 149-187, Plenum, (1986)).

Replication-deficient retroviruses are capable of directing synthesis of all virion proteins, but are incapable of making infectious particles. Accordingly, these genetically altered retroviral expression vectors have general utility for high-efficiency transduction of genes in cultured cells, and specific utility for use in the method of the present invention. Such retroviruses further have utility for the efficient transduction of genes into mesothelial cells in vivo. Retroviruses have been used extensively for transferring genetic material into cells. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with the viral particles) are provided in Kriegler, M. Gene Transfer and Expression, A Laboratory Manual, W.H. Freeman Co, New York, (1990) and Murray, E.J., ed. Methods in Molecular Biology, Vol. 7, Humana Press Inc., Clifton, N.J., (1991).

The major advantage of using retroviruses for gene therapy is that the viruses insert the gene encoding the therapeutic agent into the host cell genome, thereby permitting the exogenous genetic material to be passed on to the progeny of the cell when it divides. In addition, gene promoter sequences in the LTR region have been reported to enhance expression of an inserted coding sequence in a variety of cell types (see e.g., Hilberg et al., Proc. Natl. Acad. Sci. USA 84:5232-5236 (1987); Holland et al., Proc. Natl. Acad. Sci. USA 84:8662-8666 (1987); Valerio et al., Gene 84:419-427 (1989). The major disadvantages of using a retrovirus expression vector are (1) insertional mutagenesis, i.e., the insertion of the therapeutic gene into an undesirable position in the target cell genome which, for example, leads to unregulated cell growth and (2) the need for target cell proliferation in order for the therapeutic gene carried by the vector to be integrated into the target genome (Miller, D.G., et al., Mol. Cell. Biol. 10:4239-4242 (1990)). While proliferation of the target cell is readily achieved *in vitro*, proliferation of many potential target cells *in vivo* is very low.

Despite these apparent limitations, *in vivo* gene therapy using replication-deficient retroviral vectors to deliver a therapeutically effective amount of a therapeutic agent can be efficacious if the efficiency of transduction is high and/or the number of target cells available for transduction is high. Methods for stimulating mesothelial cell proliferation *in vivo* have been reported (Aronson, J.F. et al. Lab. Invest. 34:529-536 (1976)) and can be adapted to increase the number of target mesothelial cells *in vivo*. Accordingly, the potentially large number of mesothelial cells available for *in vivo* gene therapy, (e.g., ~1 to 4 x 10^9 cells in the adult human, based on the observed confluent cell culture density of ~1 to 3 x 10^5 cells/cm^2 and a peritoneal surface area of ~$1m^2$ (Esperanca, M.J. and Collins, D.L. J. Ped. Surg. 1:162-169

(1966); Rubin, B.J. et al., Am. J. Med. Sci. 295:453-458 (1988)) as well as the large area available for implantation of extracorporeally transformed mesothelial cells (e.g. ~$1m^2$ in the adult human (Esperanca, M.J. and Collins, D.L. J. Ped. Surg. 1:162-169 (1966); Rubin, B.J. et al., Am. J. Med. Sci. 295:453-458 (1988)), represent substantial advantages for the use of the mesothelial cells as target cells for human gene therapy.

Yet another viral candidate useful as an expression vector for transformation of mesothelial cells is the adenovirus, a double-stranded DNA virus. The adenovirus is frequently responsible for respiratory tract infections in humans and thus appears to have an avidity for the epithelium of the respiratory tract (Straus, S., The Adenovirus H.S. Ginsberg, Editor, Plenum Press, New York, p. 451-496 (1984)). Moreover, the adenovirus is infective in a wide range of cell types, including, for example, muscle and endothelial cells (Larrick, J.W. and Burck, K.L., Gene Therapy, Application of Molecular Biology, Elsevier Science Publishing Co., Inc., New York, p. 71-104 (1991)). The adenovirus also has been used as an expression vector in muscle cells in vivo (Quantin, B., et al., Proc. Natl. Acad. Sci. USA 89:2581-2584 (1992)).

Like the retrovirus, the adenovirus genome is adaptable for use as an expression vector for gene therapy, i.e., by removing the genetic information that controls production of the virus itself (Rosenfeld, M.A., et al., Science 252:431-434 (1991)). Because the adenovirus functions in an extrachromosomal fashion, the recombinant adenovirus does not have the theoretical problem of insertional mutagenesis. However, because the adenovirus functions in an extra-chromosomal fashion, adenoviral transformation of a target mesothelial cell may not result in stable transduction.

Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable viral expression vectors are available for transferring exogenous genetic material into

mesothelial cells. The selection of an appropriate expression vector to express a therapeutic agent for a particular condition amenable to gene replacement therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation.

In an alternative embodiment, the expression vector is in the form of a plasmid, which is transferred into the target mesothelial cells by one of a variety of methods: physical (e.g., microinjection (Capecchi, M.R., Cell 22:479-488 (1980)), electroporation (Andreason, G.L. and Evans, G.A. Biotechniques 6:650-660 (1988), scrape loading, microparticle bombardment (Johnston, S.A., Nature 346:776-777 (1990)) or by cellular uptake as a chemical complex (e.g., calcium or strontium co- precipitation, complexation with lipid, complexation with ligand) (Methods in Molecular Biology, Vol. 7, Gene Transfer and Expression Protocols, Ed. E.J. Murray, Humana Press (1991)). Several commercial products are available for cationic liposome complexation including Lipofectin® (Gibco-BRL, Gaithersburg, MD) (Felgner, P.L., et al., Proc. Natl. Acad. Sci. 84:7413-7417 (1987)) and Transfectam® (ProMega, Madison, WI) (Behr, J.P., et al., Proc. Natl. Acad. Sci. USA 86:6982-6986 (1989); Loeffler, J.P., et al., J. Neurochem. 54:1812-1815 (1990)). However, the efficiency of transfection by these methods is highly dependent on the nature of the target cell and accordingly, the conditions for optimal transfection of nucleic acids into mesothelial cells using the above-mentioned procedures must be optimized. Such optimization is within the scope of one of ordinary skill in the art without the need for undue experimentation.

The instant invention also provides various methods for making and using the above-described genetically-modified mesothelial cells. In particular, the invention provides a method for genetically modifying mesothelial cell(s) of a

mammalian recipient ex vivo and administering the genetically modified mesothelial cells to the mammalian recipient. In a preferred embodiment for ex vivo gene therapy, the mesothelial cells are autologous cells, i.e., cells isolated from the mammalian recipient. As used herein, the term "isolated" means a cell or a plurality of cells that have been removed from their naturally-occurring in vivo location. Methods for removing mesothelial cells from a patient, as well as methods for maintaining the isolated mesothelial cells in culture are known to those of ordinary skill in the art (Stylianou, E., et al., Kidney Intl. 37:1563-1570 (1992); Hjelle, J.H., et al., Peritoneal Dialysis Intl. 9:341-347 (1989); Heldin, P. Biochem. J. 283:165-170 (1992); Di Paolo, N., et al., Int. J. Art. Org. 12:485-501 (1989); Di Paolo, N., et al., Clinical Nephrol. 34:179-1848 (1990); Di Paolo, N., et al., Nephron 57:323-331 (1991)).

The instant invention also provides methods for genetically modifying mesothelial cells of a mammalian recipient in vivo. According to one embodiment, the method comprises introducing an expression vector for expressing a heterologous gene product into mesothelial cells of the mammalian recipient in situ by, for example, injecting the vector into a coelomic cavity of the recipient. In a preferred embodiment, the method comprises introducing a targeted expression vector, i.e., a vector having associated therewith a molecule that is specifically recognized by the target mesothelial cell. Such targeting is conferred to the vector by, for example, using a viral vector for targeting a mesothelial cell having on its surface viral receptors which specifically recognize and associate with the virus.

In a preferred embodiment, the preparation of genetically modified mesothelial cells contains an amount of cells sufficient to deliver a therapeutically effective dose of the therapeutic agent to the recipient in situ. The determination of a therapeutically effective dose of a

specific therapeutic agent for a known condition is within the scope of one of ordinary skill in the art without the need for undue experimentation. Thus, in determining the effective dose, one of ordinary skill would consider the condition of the patient, the severity of the condition, as well as the results of clinical studies of the specific therapeutic agent being administered.

If the genetically modified mesothelial cells are not already present in a pharmaceutically acceptable carrier they are placed in such a carrier prior to administration to the recipient. Such pharmaceutically acceptable carriers include, for example, isotonic saline and other buffers as appropriate to the patient and therapy.

The genetically modified cells are administered by, for example, intraperitoneal injecting or implanting the cells or a graft or capsule containing the cells in a mesothelial cell-compatible site of the recipient. As used herein, "mesothelial cell-compatible site" refers to a structure, cavity or fluid of the recipient into which the genetically modified cell(s), mesothelial cell graft, or encapsulated mesothelial cell expression system can be implanted, without triggering adverse physiological consequences. Representative mesothelial cell-compatible sites include, for example, the peritoneal, pleural and pericardial cavities. Preferably, the mesothelial cell-compatible site communicates with the lymphatic system, thereby enabling delivery of the therapeutic agent to the vascular system.

In a preferred embodiment, the mesothelial cell-compatible site is denuded prior to implanting the cells. Exemplary denuding methods include but are not limited to: (1) injection of distilled water into the peritoneal cavity for 20 minutes, followed by scraping off a portion of the mesothelial layer; (2) injection of 0.1 % buffered trypsin for 20 minutes followed by scraping; (3) removal of mesothelial cells by gentle scraping with a cell

scraper and (4) touching a piece of Gelfilm (Upjohn, Kalamazoo, MI) to the mesothelium. The preferred denuding method is disclosed in the Examples.

The genetically modified mesothelial cells are implanted in a mesothelial cell-compatible site, alone or in combination with other genetically modified mesothelial cells. Thus, the instant invention embraces a method for modifying the mesothelial system of a recipient by using a mixture of genetically modified mesothelial cells, such that a first modified cell expresses a first therapeutic agent and a second modified cell expresses a second therapeutic agent. Other genetically modified cell types (e.g., hepatocytes, smooth muscle cells, fibroblasts, glial cells, endothelial cells or keratinocytes) can be added, together with the genetically altered mesothelial cells, to produce expression of a complex set of introduced genes. Moreover, more than one recombinant gene can be introduced into each genetically modified cell on the same or different vectors, thereby allowing the expression of multiple therapeutic agents by a single cell.

The instant invention further embraces a mesothelial cell graft. The graft comprises a plurality of the above-described genetically modified cells attached to a support that is suitable for implantation into a mammalian recipient. The support can be formed of a natural or synthetic material. In another embodiment, the graft comprises a patch of peritoneum. Accordingly to this embodiment, the support is the naturally-occurring matrix that holds the plurality of genetically modified cells together. Alternatively, the graft comprises a plurality of the above-described cells attached to a substitute for the naturally occurring matrix (e.g., Gelfoam (Upjohn, Kalamazoo, MI), Dacron, Gortex®).

According to another aspect of the invention, an encapsulated mesothelial cell expression system is provided. The encapsulated system includes a capsule suitable for

implantation into a mammalian recipient and a plurality of the above-described genetically modified mesothelial cells contained therein. The capsule can be formed of a synthetic or naturally-occurring material. The formulation of such capsules is known to one of ordinary skill in the art. In contrast to the mesothelial cells which are directly implanted into the mammalian recipient (i.e., implanted in a manner such that the genetically modified cells are in direct physical contact with the mesothelial cell-compatible site), the encapsulated cells remain isolated (i.e., not in direct physical contact with the site) following implantation. Thus, the encapsulated mesothelial system is not limited to a capsule including genetically-modified nonimmortalized mesothelial cells, but may contain genetically modified immortalized mesothelial cells.

INTRODUCTION TO EXAMPLES

As described above, the present invention provides methods for forming a mesothelial cell expression system for expressing a heterologous gene product (e.g., a therapeutic agent) in a mammalian recipient, the expression system produced thereby and pharmaceutical compositions containing the same. The following Examples are directed to demonstrating the feasibility of mesothelial cell gene therapy in a rat model system (Examples, Part A); the stable expression of transduced and transfected genes in a rat model system (Examples, Part B); preliminary results demonstrating transfection of human mesothelial cells *in vitro*, and reimplantation of the genetically modified human mesothelial cells as xenografts into nude rats (Examples, Part C); and prophetic examples relating to the use of human mesothelial cells for gene therapy (Examples, Part D).

Briefly, the Examples demonstrate that rat mesothelial cells can be readily isolated from the peritoneum and expanded *in vitro* to large cell numbers. The cultured rat peritoneal mesothelial cells were either transduced *in vitro*

with a marker gene (ß-galactosidase) using a retroviral vector or transfected in vitro using strontium phosphate co-precipitation and the plasmid pSVTKgh (hgh) and the resultant transduced or transfected mesothelial cells were expanded in cell culture and reimplanted in syngeneic rat recipients. The reimplanted transduced or transfected cells continued to express ß-galactosidase or hgh in vivo. The Examples also demonstrate that primary human mesothelial cells can be isolated, propagated in vitro, transfected with the gene for human growth hormone and implanted in nude rat recipients.

In the Examples, unless otherwise specified, restriction enzyme digests, ligations, transformations, and other routine procedures are performed as described in Molecular Cloning, A Laboratory Manual by Maniatis et al. All references, patents, and patent publications disclosed throughout this application are incorporated in their entirety herein by reference.

EXAMPLES

PART A The feasibility of mesothelial cell gene therapy in a rat model system.

1. Transduction of a rat mesothelial cell line with BAG vector.

a. Stable transduction of a rat mesothelial cell line. The Escherichia coli lacZ gene has been used as a convenient reporter gene because its product, ß-galactosidase, can be readily detected in situ through the use of histochemical assays that stain the cytoplasm of the cell blue (Sanes, J.R. et al., Embo. J. 5:3133-3142 (1986)). 4/4RM.4, a rat pleural mesothelial cell line was transduced with the BAG virus (containing the genes for ß-galactosidase and neomycin resistance) (Price, J. et al., Proc. Natl Acad. Sci. USA 84:156-160 (1987)). This was accomplished by centrifugation concentration of virus from the conditioned medium from a producing culture of psi-2 BAG cells, and

infecting subconfluent 4/4RM.4 cells with a mixture of the virus in Polybrene (Cepko, C. Lineage Analysis and Immortalization of Neural Cells via Retrovirus Vectors, in Neuromethods, Vol 16: Molecular Neurobiological Techniques, Boulton, A., Baker G.B. and Campagnoni, A.T., editors, The Humana Press, Clifton, N.J. (1989), pp. 177-219). The cells were selected in G418 ("G418-sulfate", Gibco-BRL geneticin), a neomycin analog, to yield three sublines: MB1, MB2, MB3. Each of these sublines was analyzed for ß-galactosidase activity by incubation with the ß-galactosidase substrate 5-bromo-4-chloro-3-indolyl-ß-galactoside (X-gal) (Cepko, C., supra). From 50-70% of the cells in each subline showed positive staining (blue color) with MB3> MB2 > MB1.

MB3 was subcloned using the cloning ring method by picking eight colonies and screening each colony for ß-galactosidase activity. Three subclones, MB3.1, MB3.2 and MB3.3 were selected for expansion. Further testing of subclones MB3.1, MB3.2 and MB3.3 indicated that > 95% of the cells in each subclone demonstrated ß-galactosidase activity (MB3.1> MB3.2 >MB3.3); and only a small percentage (3-5%) of the cells did not show any positive staining. Production of a blue color demonstrated the presence of ß-galactosidase activity. Subclones MB3.2 and MB3.3 were frozen down and subclone MB3.1 expanded in large scale culture. These MB3.1 cells were used in initial reimplantation studies.

2. Reimplantation of transduced rat mesothelial cell line in vivo (Example Table 1)

a. Time Course of Transduced Gene Expression. MB3.1 cells were used in the initial studies on the reimplantation of transduced mesothelial cells into syngeneic Fisher rats. The experiments were of three types: 1) six animals received MB3.1 (1×10^7 cells) i.p. These animals were sacrificed on days 2, 5, 7, 10, 14, 18 following reimplantation and at each time point the peritoneum was subjected to X-gal staining to determine if the MB3.1 cells

were able to implant on the intact peritoneal surface; 2) six animals were treated surgically to open the abdominal wall along the midline. The peritoneal surface was wounded by touching a 2 cm^2 square piece of Gelfilm (Upjohn, Kalamazoo, MI) to the mesothelium, thereby removing a section of mesothelial cells (Riese K.H., et al., Path. Res. Pract. 162:327-336 (1978)). The peritoneal walls then were sutured closed and the animals received an injection of MB3.1 (1 x 10^7 cells) i.p. These animals were sacrificed on days 2, 5, 7, 10, 14, and 18 after MB3.1 reimplantation and the peritoneum of each animal was subjected to X-gal staining to determine if MB3.1 cells preferentially implant on the denuded peritoneal surface; and 3) six animals were treated surgically (wounded with Gelfilm) as above but did not receive MB3.1 cells, rather they received an injection of HBSS alone. These animals were sacrificed at the time points listed above and were subjected to X-gal staining to monitor mesothelial wound healing in the absence of MB3.1 cells. Subsequently, a second series of animals were wounded with Gelfilm and then received MB3.1 (1 x 10^7 cells) i.p. These animals were sacrificed on days 21, 28, 35, 42, 49, 60 following reimplantation and the peritoneum of each rat was subjected to X-gal staining to analyze for expression of the reporter gene.

Excised peritoneal walls were fixed for 15 min in 0.5% glutaraldehyde, rinsed in PBS containing 2 mM $MgCl_2$ and stained with X-gal for 3 hr. As judged by the positive ß-galactosidase staining (blue color), mesothelial cells reimplanted on the surface of the Gelfilm wounded area and along the mid-line incision but not on the normal (nondenuded) peritoneal surface. A portion of the peritoneal wall showing positive ß-galactosidase staining (blue color), was dehydrated and embedded in paraffin. Four micron sections were cut, mounted and either stained with Hematoxylin & Eosin (H&E) or mounted without H&E staining. Transduced mesothelial cells that had attached to the

peritoneal surface appeared as a turquoise blue monolayer. Macrophages expressing endogenous ß-galactosidase activity were visible, as cells containing punctate-light blue staining, below the mesothelial surface. Further results of this series of reimplantation studies are summarized in Example Table 1. Taken together, these results indicate that the transduced mesothelial cells reimplanted preferentially on a denuded peritoneal surface, and that expression of the reporter gene could be detected for at least two months.

Expression of ß-galactosidase activity in Gelfilm-wounded animals was strongly positive for ~ three weeks and then variable during the period from 28-60 days post implantation. These results could be due to several possibilities: 1) lack of reimplantation of the MB3.1 mesothelial cells, 2) rejection of the implanted MB3.1 mesothelial cells or 3) successful reimplantation of the MB3.1 mesothelial cells followed by suppression of ß-galactosidase gene expression in the reimplanted mesothelial cells. These possibilities were investigated further.

b. Different time periods following trypsinization of MB3.1 cells. To monitor the extent of mesothelial denudation, three additional types of wounds were investigated: 1) injection of distilled water into the peritoneal cavity for 20 min. followed by scraping off of a portion of the mesothelial layer, 2) injection of 0.1% buffered trypsin for 20 min. followed by scraping, and 3) removal of mesothelial cells by gentle scraping with a cell scraper. The animals were sacrificed three days after surgery and the peritoneal walls were removed and stained with X-gal. Only the animal treated with trypsin showed any blue areas and these areas were small in surface area. When this experiment was repeated, the trypsin-treated animals and the distilled water-treated animal showed large blue areas (i.e., stained positively for ß-galactosidase). This inconsistent result suggested to us that the cells were not

Example Table 1.

MB3.1 Reimplantation Studies in vivo - Results of X-gal Staining

Treatment:	Days after inoculation 2	5	7	11	14	18	21	28	35	42	49	60
I.MB3.1 No wound	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND
II.MB3.1 Gelfilm wound	++	++	++	++	++	++	-	++	-	-	++	+
III. ------- Gelfilm wound only	+*	+*	+*	+*	-	ND	ND	ND	ND	ND	ND	ND

Note: - = no staining; ++ = blue staining; +* = macrophage staining; ND = Not Determined.

in the same condition prior to injection in each experiment.

In the above-described experiments, the amount of time that the MB3.1 cells had been kept on ice following trypsinization and removal from tissue culture but prior to injection into the animals following surgery had not been controlled and may have varied from 20 min. to 1 1/2 hr. In the subsequent experiments described below, the amount of time allowed to elapse between surgery and injection of the MB3.1 cells into the animals was controlled. Thirty minutes was selected as a reasonable and convenient time period.

The distilled water plus scraping treatment was performed in two animals and the reimplantation efficiency was compared for cells that had been freshly prepared and injected into the denuded peritoneum 30 min following completion of surgery with cells that had been trypsinized, washed and kept on ice for 3 1/2 hours before reimplantation in a recipient rat 30 min. following its surgery. Each animal was sacrificed one day after surgery, its peritoneal walls were removed and stained with X-gal. The results were quite dramatic in that only the animal injected with the freshly isolated mesothelial cells showed positive X-gal staining. These results demonstrate that MB3.1 cells that are kept on ice for extended periods of time in HBSS lose their ability to reimplant on a denuded peritoneal surface, although the exact reason remains unknown. Accordingly, the most preferred reimplantation procedure involves reimplanting the mesothelial cells in the peritoneal cavity immediately after trypsization.

c. Dose Response. Four animals were treated surgically to open the abdominal wall along the midline. The peritoneal surface was wounded by touching a 2 cm^2 square piece of Gelfilm to the mesothelium, thereby removing a small area of mesothelial cells. The peritoneal walls were sutured closed and the animals received an i.p. injection of MB3.1 cells (1×10^7, 1×10^6, 1×10^5, or 1×10^4 cells). These animals were sacrificed three days later and the

peritoneal walls were subjected to X-gal staining to determine the number of MB3.1 cells required for implantation on the wounded peritoneal surface. Positive X-gal staining was observed in animals given 1 x 10^7 and 1 x 10^6 MB3.1 cells but not in animals given the lower doses of MB3.1 cells. Accordingly, 5 x 10^6 cells was used as the injection dose in all subsequent reimplantation studies.

PART B: Stable expression of transduced and transfected genes in a rat model system.

1. Isolation of rat primary peritoneal mesothelial cells (Figure 1)

a. Isolation of rat primary mesothelial cells. A plastic chamber device was constructed, based on a previously reported method (Hjelle, J.T. et al., Perit. Dial. Int. 9:341-347 (1989)), to isolate the mesothelial cells from the parietal peritoneal walls of Fisher rats ex vivo. Primary mesothelial cells were removed from the excised parietal peritoneal wall using enzymatic treatment. Several different kinds of enzymatic treatment (2.5% trypsin, 0.05% collagenase, 2.5% trypsin-0.02% EDTA, 0.05% trypsin-0.02% EDTA) were compared with incubation periods varying from 5 to 90 min. Enzymatic digestion with 2.5% trypsin-0.02% EDTA for 60 min., followed by gentle scraping of the peritoneal wall surface with a cell scraper yielded a phenotypically pure population of cells that attached to the plastic tissue culture dishes within 12 to 24 hr. Rat primary peritoneal mesothelial cells were cuboidal, were contact inhibited and at confluence adopted a characteristic cobblestone appearance. The nuclei and nucleoli were visible.

b. In vitro culture of rat primary mesothelial cells. The effects of different growth media on the growth of rat peritoneal mesothelial cells were compared. DME/F12, supplemented with either 10% calf serum or 15% fetal calf serum (FCS) appeared to be the best growth media for the rat primary mesothelial cells. A growth curve for rat primary

mesothelial cells is shown in Figure 1. 1×10^5 cells were seeded in T25 flasks in DME/F12 supplemented with 15% FCS. The number of cells was determined daily by trypsinization and subsequent cell counting. Rat primary mesothelial cells cultured on tissue culture plastic reached a saturation density of 1×10^5 cells/cm^2 with a doubling time of ~24 h (Figure 1).

2. Characterization of rat primary mesothelial cells (Example Table 2)

a. Immunohistochemical staining of primary mesothelial cells and of MB3.1 cells. There are no markers that unequivocally identify mesothelial cells (Chung-Welch, N., et al., Differentiation 42:44-53 (1989)). Isolated mesothelial cells were therefore subjected to immunohistochemical staining with a series of antibodies using a fluorescein-conjugated double antibody technique as previously described (Hjelle, J.T. et al., Perit. Dial. Int. 9:341-347 (1989)). Rat primary mesothelial cells, 4/4RM.4 cells and MB3.1 cells were compared with human umbilical vein endothelial cells (HUVECS) obtained from Clonetics, San Diego, CA). The results are summarized in Example Table 2. All of the mesothelial cells were positive for cytokeratins 7, 8, 18 and 19 and vimentin and negative for a specific endothelial antigen. These results indicate a pattern of staining for the primary mesothelial cells in agreement with previously published immunohistochemical results for mesothelial cells (Stylianou,E., et al., Kid. Int. 37:1563-1570 (1990); Hjelle, J.T. et al., Perit. Dial. Int. 9:341-347 (1989);and Wu, Y. J., et al., Cell 31:693-703 (1982) and distinct from that observed for the HUVECs (Chung-Welch, N., et al., Differentiation 42:44-53 (1989)).

b. Synthesis of matrix. Primary mesothelial cells were allowed to grow for extended periods after plating (2 weeks to 1 month) with weekly feeding. The cells remained contact inhibited but were observed to begin production and

Example Table 2

Summary of Immunohistochemical Staining

Antibody	Cell Type			
	4/4 RM.4	1° Mesos	MB3.1	HUVEC
anti-cytokeratin 8.12	+/-	-	-	ND
anti-cytokeratin 8.13	+	+/-	ND	ND
anti-cytokeratin 4.62	+++	+++	+++	+++
anti-cytokeratin peptide 7	+/-	+/-	ND	ND
anti-cytokeratin peptide 8	+/-	+/-	ND	+/-
anti-cytokeratin peptide 13	+/-	-	ND	ND
anti-cytokeratin peptide 14	+/-	+/-	ND	ND
anti-cytokeratin peptide 18	+/-	+/-	ND	++
anti-desmin	-	+	-	ND
anti-EGF receptor	-	+/-	ND	ND
CD31 endothelial cell	-	-	ND	+++
A-CAM	+/-	+/-	ND	ND
anti-vimentin	+++	+++	+++	+++
anti-FVIII related antigen	+	+	ND	+++ (WB)
anti-collagen I	+++	+++	+++	ND
anti-collagen III	+++	++	++	ND
anti-collagen IV	+++	-	++	ND
anti-laminin	++++	+++	++++	ND
anti-fibronectin	+++++	++++	++++	ND
anti-tenascin	+	ND	ND	ND
anti-entactin	++	-	ND	ND

ND: not determined
WB: Weibel-Palade bodies

secretion of an extracellular matrix (Example Table 2). Analysis of the matrix by immunofluorescence staining with antibodies to a number of known extracellular matrix proteins indicated that the major components of the matrix were fibronectin and laminin, in agreement with previously published reports of extracelluar matrix production by mesothelial cells (Mackay, A.M., et al., J. Cell Sci. 95:97-107 (1990) and Rennard, S.I. et al., Am. Rev. Respir. Dis. 130:267-274 (1984)).

c. Uptake of DiI-AcLDL. The uptake of diI-AcLDL (a lipophilic fluorescent dye) was analyzed in primary mesothelial cells and compared with uptake in human umbilical vein endothelial cells (HUVECs). The HUVECs appeared strongly positive indicating extensive uptake of diI-AcLDL, while primary mesothelial cells were very weakly positive indicating only slight to moderate uptake of the lipophilic fluorescent dye. This result provides further evidence that the isolated cells were mesothelial cells.

3. Transduction of rat primary mesothelial cells in vitro.

a. Generation of stable transduced primary mesothelial cells. Rat primary mesothelial cells were transduced with BAG vector as follows: Conditioned medium from psi-2 BAG cells was centrifuged to remove cells and then filtered through a 0.45 micron filter. This virus-containing media was added to subconfluent rat primary mesothelial cells. The conditioned medium was changed 3 times over a one week period. Thereafter, the cells were subjected to selection by incubation in medium containing G418. After approximately one month, most of the primary cells died in the G418 selection media; however, several colonies did appear. Twenty colonies were transferred to duplicate 24-well plates and at confluence tested for ß-galactosidase activity. Three clones (#1, 14, and 15) were selected and expanded, and the X-gal staining process was repeated. Clone 14 stained the strongest for ß-galactosidase activity and

thus, was expanded for the reimplantation studies. Greater than 95% of the Clone 14 cells stained blue (i.e., were positive for ß-galactosidase).

4. Reimplantation of transduced rat primary mesothelial cells in vivo (Example Table 3)

a. Time Course and other wounding procedures. Transduced rat primary peritoneal mesothelial cells (Clone 14) were used for the implantation studies described below. These studies paralleled the above-described MB3.1 transduced rat mesothelial cell line experiments.

The introduction and attachment of the primary mesothelial cells on peritoneal surfaces wounded by wetting with distilled water followed by scraping with a cell scraper, was compared with mesothelial cell attachment following perioneal wall injury induced by the Gelfilm wounding (described above). The results indicate that transduced rat primary mesothelial cells can attach to the peritoneal surface that has been denuded using either method (Example Table 3).

A time course study on autologous implantation of rat primary mesothelial cells transduced with BAG vector in Gelfilm wounded recipients was performed as follows. A portion of the mesothelium covering the parietal peritoneal wall was removed by Gelfilm wounding and 5 x 10^6 rat primary mesothelial cells transduced with BAG vector (Clone 14) were injected i.p. immediately following surgery. Animals were sacrificed from one to 66 days later, and their peritoneal walls were excised. Tissues were fixed for 15 min. in 0.5% glutaraldehyde, rinsed in PBS containing 2 mM $MgCl_2$ and stained with X-gal for 3 hr. Photographs of the whole mount of the stained peritoneal walls from rats sacrificed at various times after reimplantation were taken at 4, 14 and 21 days after reimplantation. As judged by the positive ß-galac-tosidase staining (blue color), mesothelial cells reimplanted on the Gelfilm wounded surface and along the mid-line incision but not on the normal (nondenuded)

Example Table 3

Rat Primary Mesothelial Cells
Reimplantation Studies *in vivo* - Results of X-gal staining

Treatment:	Days after inoculation: 1	2	4	7	11	14	21	28	35	41	66
Distilled H_2O + Scraping	+++	+++	+++	+++	ND	++	ND	ND	ND	ND	ND
Gelfilm wounding	+++	+++	+++	+++	+++	+++	++	+	+	+/-	+/-

Note: +++ = intense blue staining
++ = less intense but more than moderate staining
\+ = moderate staining
\- = no staining
ND = Not Determined

peritoneal surface. Reimplanted transduced mesothelial cells continued to express the ß-galactosidase transgene for at least 21 days.

5. Analysis using DiO-labelled rat primary peritoneal mesothelial cells (Clone 14).

The results shown in Example Table 3 demonstrate that *in vivo* expression of ß-galactosidase in the implanted cells was initially (days 1-14) very high, tapered off (days 14-28), was moderately detectable (days 28-66) and ultimately was undetectable. In contrast, Clone 14 cells in culture exhibited a relatively constant or slightly decreasing level of expression which extended to passage 25 (i.e., >175 days). There are at least two possible explanations for the observed inconsistency in Clone 14 expression *in vivo* vs. *in vitro*: 1) cells that had initially attached to the denuded peritoneum subsequently sloughed off by day 28, and/or 2) implanted cells attached and remained attached throughout the observation period but the expression of the *lac*Z gene then was silenced. Clone 14 cells (rat mesothelial cell transduced with BAG) that had been additionally labelled *in vitro* with a lipophilic fluorescent oxacarbocyanine dye, DiO (Molecular Probes) were implanted to further investigate these possibilities.

DiO [3,3'-dioctadecyloxacarbocyanine perchlorate ("DiO"/DiOC18 (Cheng, H. et al. (1972) *J. Histochem. Cytochem.* 20:542), Molecular Probes, Eugene, OR] is a reddish-brown crystalline powder which is orange in solution (when dissolved in DMSO:Ethanol, 1:9). DiO fluorescence is bright green with absorption and emission maxima at 489 nm and 499 nm, respectively, and thus DiO can be viewed with FITC filters. DiO has been used as a marker for cell transplantation studies (Penza R. et al. (1992) *BioTechniques* 13:580; Ragnarson B. et al. (1992) *Histochemistry* 97:329; Soriano H. et al. (1992) *Transplantation* 54:717) and is nontoxic to cultured cells. Thus, cells can be stained *in*

vitro with DiO prior to cellular transplantation *in vivo* and cells stained with DiO can be clearly identified in the recipient tissue by fluorescense microscopy or flow cytometry, thereby allowing both qualitative and quantitative assessment of the success and longevity of engraftment following implantation.

Adherent cells were labelled by incubation in media supplemented with DiO as follows: A stock solution of 0.1% (w/v) DiO was made by dissolving DiO crystals in a 1:9 mixture of dimethyl sulfoxide (DMSO) and 100% ethanol. This solution was sonicated to dissolve all of the DiO crystals. This stock solution was added to culture media to result in a a final concentration ranging from 20 ng/ml to 40 µg/ml (typically 20µg/ml). The cells were cultured in the dye-containing media for several hours to several days (typically overnight). (The dose and time course are optimized for a particular cell type according to methods known to one of ordinary skill in the art). Thereafter, the dye-containing media was removed and replaced with normal media and the cells were viewed by fluorescence microscopy to confirm complete labelling. Labelled cells that were intended for transplantation studies were trypsinized, washed, and counted prior to inoculation *in vivo*. Depending on the turnover time of the plasma membrane and the cellular proliferation rate *in vivo*, the dye may be detectable for up to several weeks after transplantation.

When confluent cultures of Clone 14 cells were labelled with DiO as described above, Clone 14 cells took up the DiO dye into their membranes and remained fluorescent *in vitro* for at least 28 days. DiO-labelled Clone 14 cells ($5x10^6$-$1x10^7$) were implanted in Gelfilm wounded rats using the above-described protocol. When these rats were sacrificed at various time intervals following implantation, patches of fluorescent cells corresponding to the Gelfilm wounded area were observed for at least 93 days, indicating that the implanted mesothelial cells remained attached to the denuded peritoneum for at least three months.

In the experiments in which we marked the BAG ("ß-gal") containing mesothelial cells with DiO and then followed the presence of the cells _in vivo_, we confirmed that the presence of fluorescent cells is observed for longer times than ß-galactosidase expression, consistent with our hypothesis that the viral LTR is being shut off in some manner. Other investigators have traced a similar loss of BAG ß-gal expression to methylation of the viral LTR that controls the ß-gal gene (MacGregor, G.et al. (1987) _Som. Cell Mol. Genet._ 13:253). Although we have not yet tested for the presence of methylation directly, it is likely that methylation may play a role in the shut-down of ß-gal expression in this system.

6. Optimization of ex vivo gene transfer to rat mesothelial cells.

a. Strontium phosphate transfection. In preliminary studies, a toxic response of rat primary mesothelial cells to calcium phosphate was observed, thus precluding the use of calcium phosphate as a means to transfect rat mesothelial cells without further study. To circumvent this difficulty, a modification of the traditional calcium phosphate transfection procedure, namely the substitution of strontium phosphate for calcium phosphate, was used to transfect the rat primary mesothelial cells. The substitution of strontium phosphate for calcium phosphate has been shown to achieve stable transfection of primary cells which exhibit sensitivity to calcium phosphate (Brash. D.E., et al. _Molec. Cell. Biol._ 7:2031-2034 (1987)).

The strontium phosphate co-transfection protocol was tested on the rat mesothelial cells using pSVTKgH as a reporter. pSVTKgH is a plasmid which contains the gene for human growth hormone (hgh) driven by the thymidine kinase promoter and SV40 enhancer. The secretion of human growth hormone into the conditioned media by the transfected rat mesothelial cells was measured 2-3 days after the transfection by a solid-phase, two-site radioimmunoassay for

human growth hormone using a commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). This assay can detect as little as 0.2 ng of hgh per milliliter, and is linear in the range of 0.2 to 50 ng/ml.

The results indicate that stable transfection of rat mesothelial cells can be achieved using strontium phosphate transfection. Optimization of the transfection parameters for transfection of the rat mosothelial cells included varying the incubation time of the cells with the strontium phosphate-DNA precipitate, the pH of the transfection solution, the percent serum present in the media during the transfection and the inclusion or omission of a final glycerol shock step. The preferred protocol for the transfection of rat mesothelial cells is described below and in Brash D. et al. (1987) Molec. Cell. Biol. 7:2031.

Cells were plated in 60 mm dishes the day before transfection at approximately 25% confluence. (The cells should be at approximately 50% confluence by the day of transfection). Prior to transfection the cells were washed once with 2 ml. of medium lacking serum, followed by placing the cells under 2 ml. of medium containing 0.5% FCS. The following reagents then were prepared: Tube A (250 ml of 2X HBS solution (Hepes buffered saline (Brash et al.) pH 7.9) and Tube B (240 ml dH2O); plasmid DNA, 3 mg total; 12 ml 2M $SrCl_2$). Tube B was added to tube A dropwise while constantly flicking tube A. Immediately, thereafter, the combined volume in tube A was added to the cells dropwise using a 1 ml pipette. The cells were carefully observed to determine whether the resultant precipitate had the appearance of pepper and was just visible at 100x final magnification. If the precipitate is not visible or is present in large clumps, the transfection will not work and the above-described procedure should be repeated to optimize the DNA concentration or the $SrCl_2$ volume. When the correct size precipitate was obtained, the plates were incubated at room temperature in the hood (no CO_2) for

20-30 minutes. At the end of this time, the precipitate was observed to have settled onto the cells. The dishes were placed in an incubator and incubated for an additional 7 to 16 hours. Two days later (if the cells were still sub-confluent), the medium was replaced with growth medium supplemented with 100 µg/ml G418-sulfate. The cells were passaged when confluent and colonies appeared 7-12 days thereafter. Further optimization of the DNA concentration and strontium chloride volume may be required depending on the size of the particular plasmid construct used for transfection.

7. Stable transfection of rat mesothelial cells with pSVTKgh (Figure 3).

a. Analysis of secretion of hgh by transfected rat mesothelial cells in vitro. The plasmids pCDneo and pSVTKgh were cotransfected into rat primary peritoneal mesothelial cells using the above-described strontium phosphate coprecipitation protocol. Transfected cells were selected for survival in the presence of G418 and individual clones were screened for hgh production using a commercially available two-site radioimmunoassay for human growth hormone (Nichols Institute Diagnostics, San Juan Capistrano, CA). Three positive clones were expanded and rescreened. The clone (gh 2-3) which produced the highest levels of hgh was expanded for use in the reimplantation studies described below.

Production of hgh in vitro by the transfected rat mesothelial cells (clone gh 2-3) was analyzed approximately weekly, in conjunction with each set of reimplantation experiments. The results for the in vitro expression of hgh by these rat mesothelial cells are depicted in Figure 3. Cultures of the hgh-transfected rat mesothelial cells, when grown to confluence in 15 cm dishes, secreted hgh into the medium at the rate of ~10,000 to 24,000 ng/culture per day or an average ~1700 ng per 10^6 cells per day (or 70 ng/hr

per 10^6 cells). Production of hgh by the rat mesothelial cells fell slightly over time over the two month observation period (see Figure 3).

8. Implantation studies with pSVTKgh-transfected rat mesothelial cells (Figures 4A & 4B).

a. Analysis of secretion of hgh by transfected rat mesothelial cells in vivo. The hgh-transfected rat mesothelial cells were used in reimplantation experiments to determine the level of secretion of hgh by the reimplanted cells in vivo. The experiments were conducted as follows. Fisher rats were anesthetized with sodium pentobarbital and a midline incision was made in the abdominal skin and underlying peritoneal wall. The peritoneal surface of these animals was denuded by Gelfilm wounding. To maximize the surface area available for reseeding by the hgh-transfected mesothelial cells, 4 pieces of gelfilm were used. The surface area of each piece of Gelfilm was 2 cm x 2 cm; therefore, the total surface area of the peritoneal wall that was denuded was at most ~16 cm^2. The abdominal cavity was sutured closed and each rat received an iNpN injection of ~1 x 10^7 hgh-transfected mesothelial cells per animal (range: 5 x 10^6 to 2 x 10^7 cells) in 2 ml of HBSS.

To determine whether the transfected mesothelial cells attached to the denuded peritoneal wall would secrete hgh into the peritoneal fluid which would be taken up by the lymphatics and ultimately reach the bloodstream, eyebleeds were performed by retroorbital puncture. Blood was taken into a known volume of heparin at various timepoints after introduction of the transfected cells (4, 8, 12, 24, 48 hr and then weekly) to obtain blood (and then plasma by subsequent centrifugation) for hgh analysis. The levels of hgh in the plasma were determined using the above-identified two-site radioimmunoassay for human growth hormone (Nichols Institute Diagnostics, San Juan Capistrano, CA). The results of these assays are shown in Figures 4A & 4B.

The time course of hgh expression by the implanted cells was divided into several phases. First, ~1 ng hgh/ml blood was detected within 4 hr of surgery. The level of hgh in the plasma appeared to rise thereafter, peaking at ~7 ng/ml by 6 hr, and then declined to ~3.5 ng/ml by 12 hrs and to ~0.66 ng/ml by 24 hrs. Thereafter, the level of hgh fell off rapidly and was indistinguishable from the level of hgh in control animals by day ~4-7. An immunological response to hgh is believed to play a role in the decline in detectable hgh with increasing time (discussed below).

The theoretically expected steady state hgh concentration in rat serum can be calculated as follows (Teumer J. et al. (1990) FASEB J. 4:3245): equilibrium concentration = [(hgh production from the graft in ng/h)/equilibrium volume]/K. $K=\ln 2/T_{1/2}$. $T_{1/2}$ was assumed to be 4 min. (Peeters S. et al. (197) Endocrinology 101:1164). The equilibrium volume was assumed to be 22% of the rat body weight (Teumer J. et al. (1990) FASEB J. 4:3245) (i.e., 37.4 ml in a 170g rat). Therefore, based on an *in vitro* production rate of 70 ng/hr/10^6 cells, and on the number of transfected mesothelial cells reseeded on the denuded peritoneum being equal to 1.6 to 4.8 x 10^6 cells (based on a mesothelial cell saturation density of 1 to 3 x 10^5 cells/cm^2 and an available denuded surface area of 16 cm^2), an expected equilibrium steady-state hgh concentration of 0.3 to 0.9 ng/ml in rat plasma was calculated. This calculation assumes: 1) that the *in vivo* rate of hgh production by the mesothelial cells was equal to that observed *in vitro*; 2) that there was no interference by an immune response; and 3) that coverage of the denuded surface was 100%.

The results indicated that an hgh level of 0.6 ng/ml was obtained within one day of surgery (transient expression was often higher at ~7 ng/ml), but that this level of detectable hgh did not persist. The day one hgh level of 0.6 ng/ml fell within the range of the theoretically calculated

steady state hgh concentration. The results demonstrate that intraperitoneal implantation of stably transfected mesothelial cells can deliver a detectable protein product to the bloodstream.

9. Limitations of introduced gene (hgh) expression in the rat mesothelial cell model system.

a. Interference of the measurable hgh levels in rats by a substance in sensitized rat plasma. The serum from Fisher rats implanted with hgh-secreting mesothelial cells, when admixed with a standard amount of hgh standard, interfered with the detectability of the hgh standard (see Bennett and Chang (1990) Molecular and Biol. Medicine 7:471). The time course for the appearance of this "interfering activity" is shown in Figures 5A and 5B.

b. The identity of the interfering material: presence of hgh antibodies in the plasma of animals reimplanted with hgh-secreting rat mesothelial cells. An inhibitory substance which interfered with the measurement of hgh levels using a commercial RIA kit was detected in the plasma of control (nonimmunosupressed) rats implanted with hgh-secreting mesothelial cells. The titer of this interfering substance increased with time and it was suspected that the substance was an antibody. A sensitive immunoblotting methodology was used to establish the presence of anti-hgh antibodies in the plasma of these rats. Anti-hgh antibody levels in the rat plasma samples were undetectable on days 1 and 4 following mesothelial cell implantation, became detectable by day 29, increased with time (day 50) and were present at high levels at 64 days. The fact that these immunocompetent rats had mounted an immune response which increased with time suggests that hgh secretion by the reimplanted mesothelial cells in these animals persists for at least two months following implantation. The existence of an additional inhibitory substance, e.g., an hgh binding protein, has not been excluded. A high affinity binding

protein of growth hormone which increases in titer in response to increased growth hormone production has been reported (Baumann G. et al. (1986) J. Clin. Endocrinol. Meta. 62:134). Further assay inhibition and other binding studies can be performed to determine whether the hgh interfering activity is solely attributable to an immune system response or whether an hgh binding protein may also play a role in inhibition of detectable hgh in plasma.

10. Secretion of hgh in immunosuppressed animals (Figure 6).

To circumvent possible antibody production, the immune systems of the recipient animals were suppressed to allow detection of hgh secretion by the reimplanted, genetically-modified mesothelial cells and in particular, to observe the long-term expression of hgh in the systemic circulation. Chemical immunosuppression was achieved by placing dexamethasone (Sigma Chemical Co., St. Louis, MO)(1.2 mg/l) in the animals' drinking water (Reddy L. et al. (1991) J. Protozool 38:45S). Tetracycline-HCl (Sigma) at 0.5 g/l also was added to the drinking water of dexamethasone-treated animals to prevent bacterial infection. Treatment was begun 4 days prior to the surgery for mesothelial cell reimplantation and continued throughout the subsequent hgh measurements.

The same transient phase increase (6-24 hr) in hgh levels in plasma was observed for the control and immunosuppressed animals; however, the level of hgh in the plasma of immunosuppressed animals did not drop as rapidly, but rather reached a steady-state plateau at ~0.1 ng/ml. This plasma hgh concentration persisted for ~2-3 weeks (Figure 6).

The theoretically expected steady state hgh concentration in rat serum was calculated as described above. An equilibrium steady-state hgh (minimum) concentration of ~0.3 ng/ml in rat plasma was expected.

This calculation assumed: 1) that the *in vivo* rate of hgh production by the mesothelial cells was equal to that observed *in vitro*; 2) that there was no interfering activity and 3) that coverage of the denuded surface was 100%. However, because Gelfilm denudation is not perfect and subsequent coverage is incomplete, an ~50% coverage was estimated to calculate the lower limit for the steady state hgh concentration. Using this coverage estimate, a steady state plasma hgh concentration of ~0.15 ng/ml was estimated. This value was in fairly good agreement with the observed hgh plasma level. The decrease in detectable hgh expression with increasing time may represent promoter silencing such as that described above in regard to LTR-driven ß-gal expression.

11. Optimization of denudation/exfoliation procedures using BAG-transduced mesothelial cells (Example Table 4).

a. Denudation by Gelfilm wounding. Preliminary studies indicated that the peritoneal wall must be denuded in order for the transfected/transduced mesothelial cells to attach following i.p. injection. Typically this was accomplished by Gelfilm wounding (Cheng, H. et al. (1972) *J. Histochem. Cytochem.* 20:542). However, the removal of mesothelial cells by the Gelfilm sometimes can be incomplete. Accordingly, the number of 2 cm x 2 cm pieces of Gelfilm used per animal was increased from one to two to four to provide a larger available surface area for mesothelial cell attachment. Multiple pieces of Gelfilm at the same site also have been used to more thoroughly denude the area. These modifications of the Gelfilm wounding procedure improved denudation.

b. Denudation by other wounding procedure. To ensure the complete removal of mesothelial cells, several alternative denudation protocols were investigated. Mesothelial cells are extremely sensitive even to minor

injury. Mild drying or wetting of rat caecal peritoneum for five minutes has been reported to induce mesothelial cell degeneration and detachment (Ryan G. et al. (1973) Am. J. Pathol. 71:93; Ryan G. et al. (1971) Am. J. Pathol. 65:117). Accordingly, the following alternatives to Gelfilm wounding were tested: 1) drying by a stream of air; 2) drying by filter paper blotting; 3) wetting with isotonic saline; 4) wetting with PBS; 5) wetting with 100% ethanol; 6) wetting with 95% ethanol; 7) wetting with 70% ethanol; and 8) wetting with DMSO. Wetting with ethanol or distilled H_2O, followed by scraping also has been tested. The results (Example Table 4) indicate that the Gelfilm denudation protocol is the most reproducible and reliable procedure and yields the most uniform denuded surface. However, air drying of the peritoneal surface also yielded a fairly uniform patch of cells, as did wetting with isotonic saline, suggesting that these alternative procedures can be further optimized to achieve improved denudation.

PART C Preliminary results relating to the isolation of human mesothelial cells, the transfection of human mesothelial cell in vitro and the reimplantation of transfected human mesothelial cells in vivo.

1. Isolation of primary human peritoneal mesothelial cells.

Human primary mesothelial cells were isolated from discarded surgical specimens (omentum) by trypsinization (Stylianou, E., et al., Kidney Intl. 37:1563-1570 (1990); Pronk, A. et al., In Vitro Cell. Dev. Biol., 29A:127-134 (1993)). Human primary mesothelial cells were isolated from five patients as follows. Large sections of human omentum were obtained from patients (previously diagnosed with ulcerative colitis or Crohn's disease) who were undergoing ileo-anal pouch reconstruction surgery. The dissected omental tissue was collected in sterile cold HBSS in the Operating Room at the time of excision and was brought to the

Example Table 4

Rat Primary Mesothelial Cells
Reimplantation Studies in vivo -
Various Wounding Protocols

Treatment	Days after inoculation	
	1	5
Denudation Protocols:		
Gelfilm	+++	+++
Multiple Gelfilms over same area	ND	+++
Drying Protocols:		
Drying with a stream of air	+	+++
Drying with filter paper	+	
Wetting Protocols:		
Isotonic saline	+	+++
PBS	-	
100% ethanol	+	
100% ethanol + scraping	+	
95% ethanol	-	
70% ethanol	-	
DMSO	ND	+++
Distilled H_2O	+	
Distilled H_2O + scraping	+++	

ND = Not Determined
+++ = intense blue staining
++ = moderate blue staining
+ = blue staining.

laboratory on ice. The isolation procedure was begun within 30 min. of tissue removal from the patient. The intact omental tissue was first weighed to obtain a wet weight. (Average wet weight = 132g; range of wet weight = 40g to 290g) and then incubated in 200 ml trypsin-EDTA (0.05% trypsin-0.53 mM EDTA) for 20 min. at 37°C with stirring. The omental tissue was removed to a fresh 200 ml aliquot of trypsin-EDTA for another 20 min. incubation, and the dissociated cells found in this first aliquot of trypsin-EDTA were recovered by centrifugation. This process was repeated for a total of 5-6 sequential trypsinizations. After each digestion, the isolated cells were washed 3x with HBSS to remove red blood cells, the cells were resuspended in culture media and plated out in 15 cm dishes.

Putative human mesothelial cells were grown in DME/F12, 15% FCS, L-glutamine, and antibiotics in the presence of hydrocortisone (0.4 μg/ml) and EGF (epidermal growth factor)(5-10 ng/ml) (Rheinwald, J.G. "Methods for clonal growth and serial cultivation of normal epidermal keratinocytes and mesothelial cells", in Cell Growth and Division: a practical approach, Baserga, R., Editor, IRL Press, Oxford, England (1989), pp. 81-94). As identified by immunofluorescence microscopy (discuss below), human mesothelial cells typically were found in aliquots 2 and 3 of the trypsin-EDTA, while human fibroblasts were found in aliquots 5 and 6. Cultures of primary human mesothelial cells, grown in the presence of HC and EGF were expanded and frozen at passages 1 through 4. When human mesothelial cells were placed in tissue culture in media containing 5-10 ng/ml EGF and hydrocortisone (0.4 μg/ml), they grew rapidly, assumed a quasi-fibroblastoid morphology and were not contact inhibited. However, when these mesothelial cells were deprived of EGF when the culture was ~50% confluent, the growth rate of the mesothelial cells slowed substantially,

the cells became more flattened and stopped dividing when a single cell monolayer was reached, reminiscent of their *in vivo* morphology (Rheinwald, J.G, supra).

The primary mesothelial cells from five donors were expanded and frozen down at early passages, preferably at passage numbers 1 and 2. These mesothelial cells have been successfully cultured for at least 4-5 passages, without a change in phenotype, however, the percentage of large multinucleated cells increased with each passage. Cells from the first donor deteriorated completely by about passage number 10, i.e., large, multinucleated cells appeared to dominate the culture, even in the presence of EGF. Accordingly, the results indicate that gene transfer transfection procedures should be initiated relatively quickly after cells have been isolated from the donor, preferably at passages 2 or 3. The culture conditions for the primary human mesothelial cells can be optimized according to standard practice known to one of ordinary skill in the art to ensure that the human mesothelial cells intended for gene therapy use remain viable throughout the transfection, selection, and expansion process prior to implantation.

2. Characterization of primary human mesothelial cells.

Indirect immunofluorescence was used to confirm the identity of the human primary mesothelial cells. Human mesothelial cells (from passage 3 and from passage 8) stained positively with the following antibodies: anti-cytokeratin 19; anti-cytokeratin peptide 8; and anti-cytokeratin peptide 18. The human mesothelial cells stained negatively with an anti-endothelial cell antigen von Willebrand Factor, (VWF), and with anti-desmin. Taken together, these results indicate a pattern of staining for the primary human mesothelial cells that is in agreement with previously published immunohistochemical results for mesothelial cells (Stylianou,E., et al., *Kid. Int.* 37:1563-1570 (1990); Hjelle,

J.T. et al., Perit. Dial. Int. 9:341-347 (1989); and Wu, Y. J., et al., Cell 31: 693-703 (1982) and that is distinct from that observed for endothelial cells.

3. Transfection of human mesothelial cells with PSVTKgh (Figure 7).

pSVTKgH is a plasmid containing the gene for human growth hormone (gH) (Selden, R.F. et al., Mol. Cell. Biol. 6:3173-3178,1986). pSVTKgH was used as a reporter to optimize the strontium phosphate transfection protocol in primary human mesothelial cells. The mesothelial cells were cotransfected with supercoiled plasmids containing genes for growth hormone and neomycin resistance. Growth hormone expression was measured 2-3 days after the transfection using the above-described solid-phase, two-site radioimmunoassay for human growth hormone (Nichols Institute Diagnostics, San Juan Capistrano, CA). Cotransfected uncloned human mesothelial cells were found to secrete detectable amounts of human growth hormone into culture medium (Figure 7). Cultures of hgh transfected human mesothelial cells (uncloned) secreted hgh into the medium at a rate of ~160 ng per 10^5 cells per day. The results (Figure 7) indicate that human mesothelial cells can be transfected with pSVTKgH to achieve expression of a transfected gene. The latter cells were used in the following reimplantation studies in nude rats.

4. Implantation of pSVTKgh-transfected human mesothelial cells into Gelfilm wounded nude rats: Pilot Experiment with DiO-labelled cells.

The human mesothelial cells, stably transfected with pSVTKgH using the strontium phosphate method (Brash. D.E., et al., Molec. Cell. Biol. 7:2031-2034 (1987) were used to test for the ability of human mesothelial cells to attach as xenografts in nude rats. In a pilot study, a pooled population of stably transfected uncloned human mesothelial

cells, was labelled with the fluorescent cell tracker, DiO (Molecular Probes, Eugene, OR), and reimplanted into a nude rat. The pilot study included two nude rats: (1) a control animal (surgery, Gelfilm wound, no cells implanted) and (2) an experimental animal (surgery, Gelfilm wound, reimplantation of 1 x 10^5 pSVTKgH transfected, DiO-labelled human mesothelial cells). On day 18, the experimental animal was sacrificed and the peritoneal wall was examined for the presence of DiO-labelled human mesothelial cells. Small patches of fluorescent cells were observed, scattered over the peritoneal surface. These results indicate that transfected human mesothelial cells can implant on a denuded peritoneal surface and remain attached to that surface for at least 18 days.

PART D Prophetic Example of human mesothelial cell gene therapy (Figure 2)

A procedure for human gene therapy using genetically modified mesothelial cells is described herein. The preferred method utilizes autologous cells, i.e., cells that are isolated from the intended recipient of the genetically modified cells. The cells are harvested from the human donor by sampling the lining of a coelomic cavity, e.g. the surface of the omentum, according to methods known to one of ordinary skill in the art. Harvesting is performed at a time of surgical intervention or by laparoscopy. Thereafter, the harvested cells are established in cell culture according to methods known in the art for culturing mesothelial cells.

To prepare an expression vector for expressing a heterologous gene encoding a therapeutic agent, the gene is inserted into a viral vector according to methods known in the art. Alternatively, the gene can be inserted into a plasmid vector. The heterologous gene preferably includes a constitutive promoter to permit transcription of the heterologous gene following its introduction into the mesothelial cell. Additional control elements, e.g.

enhancers, are inserted into the heterologous gene according to standard methods to allow greater control of expression. Alternatively, inducible promoters are used to regulate transcription of the inserted gene. However, the use of inducible promoters further requires the step of exposing the genetically modified mesothelial cells to inducing agents in situ to achieve expression of the therapeutic agent in situ.

The expression system further includes a selectable marker (e.g., a marker for neomycin resistance) to facilitate selection of genetically modified mesothelial cells. The mesothelial cells are virally transduced or transfected in vitro with the above-described viral vector or plasmid construct according to methods known in the art. Culture of the genetically modified mesothelial cells is performed in the presence of a selection medium (e.g., a medium containing neomycin) and the genetically modified cells are characterized. Only those genetically modified cells exhibiting stable expression of the therapeutic agent at a therapeutically effective level are selected for further characterization.

The selected mesothelial cells are evaluated by immunohistochemical staining to determine whether the transduced cells are suitable for administration to the human recipient. Transduced or transfected cells suitable for direct administration to the recipient are nonimmortalized and are nontumorigenic. Additional characterization of the transduced or transfected cells is performed to establish that the cells comply with standards established by government agencies responsible for overseeing human gene therapy clinical trials.

Administration of the genetically modified mesothelial cells is by intraperitoneal injection of a suspension containing the cells into a coelomic cavity of the human recipient or by implanting the cells. Preferably, the site of implantation is denuded prior to implantation. For therapeutic agents which are directed to the systemic

circulation, successful expression of the therapeutic agent _in situ_ is evaluated by determining blood levels of the agent. In general, efficacy of the gene transfer therapy is determined by a reduction in clinical symptoms attributable to the condition for which the therapeutic agent is being administered.

It should be understood that the preceding is merely a detailed description of certain preferred embodiments. It therefore should be apparent to those skilled in the art that various modifications and equivalents can be made without departing from the spirit or scope of the invention.

CLAIMS

1. A mesothelial cell expression system for expressing exogenous genetic material in a mammalian recipient, the expression system comprising

a mesothelial cell; and

an expression vector contained therein, said expression vector for expressing the exogenous genetic material, wherein said expression system is suitable for administration to the mammalian recipient.

2. An expression system as claimed in claim 1, wherein the mammalian recipient is a human.

3. An expression system as claimed in claim 2, wherein said mesothelial cell is isolated from said human recipient.

4. An expression system as claimed in claim 3, wherein said mesothelial cell comprises a cell selected from the group consisting of a parietal mesothelial cell, a visceral surface mesothelial cell and a free-floating mesothelial cell.

5. An expression system as claimed in claim 4, wherein said mesothelial cell is isolated from the group consisting of a pleural cavity, a pericardial cavity and a peritoneal cavity.

6. An expression system as claimed in claim 1, wherein the mammalian recipient has a condition amenable to gene replacement therapy and said exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating said condition.

7. An expression system as claimed in claim 6, wherein said condition is selected from the group consisting of a genetic disease, an acquired pathology, a cancer and a prophylactic process.

8. An expression system as claimed in claim 7, wherein said condition comprises a genetic disease and wherein said exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating said genetic disease.

9. An expression system as claimed in claim 8, wherein said genetic disease is selected from the group consisting of Immunodeficiency, Hypercholesterolaemia, Haemophilia A, Haemophilia B, Gaucher's disease, Mucopolysaccharidosis, Emphysema, Phenylketonuria, Hyperammonaemia, Citrullinaemia, Muscular dystrophy, Thalassaemia, Sickle cell anaemia, Leukocyte adhesion deficiency and von Willebrand's disease.

10. An expression system as claimed in claim 8, wherein said therapeutic agent is selected from the group consisting of Adenosine deaminase, Purine nucleoside phosphorylase, LDL receptor, Factor IX, Factor VIII, Glucocerebrosidase, beta-glucuronidase, alpha1-antitrypsin, Phenylalanine hydroxylase, Ornithine transcarbamylase, Arginosuccinate synthetase, Dystrophin, beta-globin, CD-18 and von Willebrand Factor.

11. An expression system as claimed in claim 7, wherein said condition comprises an acquired pathology and said exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating said acquired pathology.

12. An expression system as claimed in claim 11, wherein said acquired pathology is selected from the group consisting of anemia, peritoneal sclerosis, peritonitis, uremia, septic shock, diabetes, pituitary Dwarfism, thrombosis, post-surgical adhesions, end stage renal disease, and AIDS.

13. An expression system as claimed in claim 11, wherein said therapeutic agent is selected from the group consisting of erythropoietin, thrombomodulin, tissue plasminogen activator, insulin, single chain urokinase plasminogen activator, superoxide dismutase, urease, thrombomodulin, insulin, human growth hormone, hirudin, catalase and CD-4.

14. An expression system as claimed in claim 7, wherein said condition comprises a cancer and said exogenous genetic material comprises a heterologous gene encoding an anti-neoplastic agent for treating said cancer.

15. An expression system as claimed in claim 14, wherein said cancer comprises a metastatic cancer of the peritoneal or pleural cavities.

16. An expression system as claimed in claim 14, wherein said anti-neoplastic agent is selected from the group consisting of a mutated oncogene expression product, an antisense RNA, a normal tumor-suppressor, a cytokine, an interferon, a tumor necrosis factor and an interleukin.

17. An expression system as claimed in claim 7, wherein said condition comprises a prophylactic process and said exogenous genetic material comprises a heterologous gene encoding a prophylactic agent.

18. An expression system as claimed in claim 17, wherein said prophylactic process is for preventing pregnancy and wherein said therapeutic agent includes estrogen and progesterone.

19. An expression system as claimed in claim 17, wherein said prophylactic process is for preventing hypothyroidism and wherein said therapeutic agent is thyroxine.

20. An expression system as claimed in claim 1, wherein said expression vector comprises a replication-deficient virus.

21. An expression system as claimed in claim 1, wherein said expression vector comprises a chimeric plasmid containing a nucleic acid encoding a therapeutic agent.

22. An expression system as claimed in claim 1, said expression vector further comprising a promoter for controlling transcription of said heterologous gene.

23. An expression system as claimed in claim 22, said expression vector further comprising a signal sequence for delivering said therapeutic agent from the mesothelial cells to an in vivo location selected from the group consisting of an extracellular milieu and a cellular membrane.

24. A pharmaceutical composition, comprising:

a plurality of genetically modified mesothelial cells, said cells containing an expression vector for expressing exogenous genetic material; and

a pharmaceutically acceptable carrier.

25. A pharmaceutical composition as claimed in claim 24, wherein said composition is for treating a condition amenable to gene replacement therapy and wherein said exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating said condition.

26. A pharmaceutical composition as claimed in claim 25, wherein said therapeutic agent is selected from the group consisting of an agent for treating a genetic disease, an agent for treating an acquired pathology, an anti-neoplastic agent for treating a cancer and a prophylactic agent.

27. A pharmaceutical composition as claimed in claim 25, wherein said composition contains an amount of cells sufficient to deliver a therapeutically effective dose of said therapeutic agent in situ to a patient receiving said composition.

28. A mesothelial cell graft, comprising,

a support suitable for implantation into a mammalian recipient; and a plurality of genetically modified mesothelial cells attached to said support.

29. A graft as claimed in claim 28, wherein said support comprises a patch of peritoneum and said mesothelial cells contain a recombinant gene.

30. A graft as claimed in claim 28, further comprising a substrate for facilitating attachment of said cells to said support.

31. A graft as claimed in claim 28, wherein said support comprises a synthetic material.

32. An encapsulated mesothelial cell expression system, comprising,

a capsule suitable for implantation into a mammalian recipient; and a plurality of genetically modified mesothelial cells contained within said capsule.

33. A method for making a pharmaceutical preparation for administration to a mammalian recipient, the method comprising the steps of:

(a) introducing an expression vector for expressing a heterologous gene product into an isolated mesothelial cell to form a genetically modified mesothelial cell; and

(b) placing the genetically modified mesothelial cell in a pharmaceutically acceptable carrier.

34. A method for genetically modifying the mesothelial system of a mammalian recipient, the method comprising:

introducing an expression vector for expressing a heterologous gene product into a mesothelial cell of the mammalian recipient in situ.

35. A method as claimed in claim 34, wherein introducing an expression vector comprises injecting said expression vector into the coelomic cavities of mammalian recipient.

36. A method as claimed in claim 34, wherein said expression vector further includes an inducible promoter for controlling transcription of said heterologous gene product, said method further comprising the step of exposing the genetically modified mesothelial cell in situ to conditions for permitting transcription of said heterologous gene product.

37. A method for genetically modifying the mesothelial system of a mammalian recipient, the method comprising:

introducing an expression vector for expressing a heterologous gene product into an isolated mesothelial cell to form a genetically modified mesothelial cell; and

administering said genetically modified mesothelial cell to the mammalian recipient.

38. A method as claimed in claim 37, wherein said mesothelial cell is isolated from the mammalian recipient.

39. A method as claimed in claim 37, wherein administering said genetically modified cell comprises injecting said genetically modified cell into said mammalian recipient.

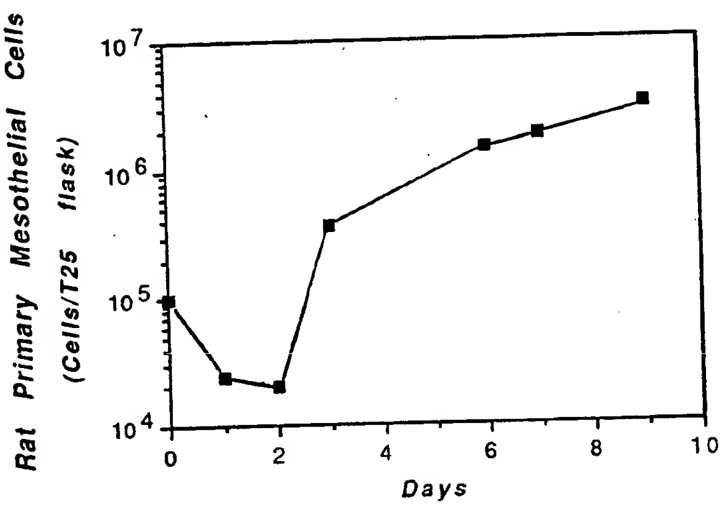
40. A method as claimed in claim 37, wherein administering said genetically modified mesothelial cell comprises implanting said genetically modified cell into a mesothelial cell-compatible site of the mammalian recipient.

41. A method as claimed in claim 40, further comprising the step of denuding the mesothelial cell-compatible site prior to implanting said genetically modified mesothelial cell.

42. A method as claimed in claim 37, wherein administering said genetically modified mesothelial cell comprises implanting a mesothelial cell graft including a plurality of said genetically modified cells attached to a support.

43. A method as claimed in claim 37, wherein administering said genetically modified mesothelial cell comprises implanting an encapsulated mesothelial cell expression system including a plurality of said genetically modified cells contained within a capsule suitable for implantation into the mammalian recipient.

44. A method as claimed in claim 37, wherein said expression vector further includes an inducible promoter for controlling transcription of said heterologous gene product, said method further comprising the step of exposing the genetically modified mesothelial cell <u>in situ</u> to conditions for permitting transcription of the heterologous gene product.



Rat Primary Mesothelial Cells
(Cells/T25 flask)
10^7
10^6
10^5
10^4
0
2
4
6
8
10
Days

Figure 1

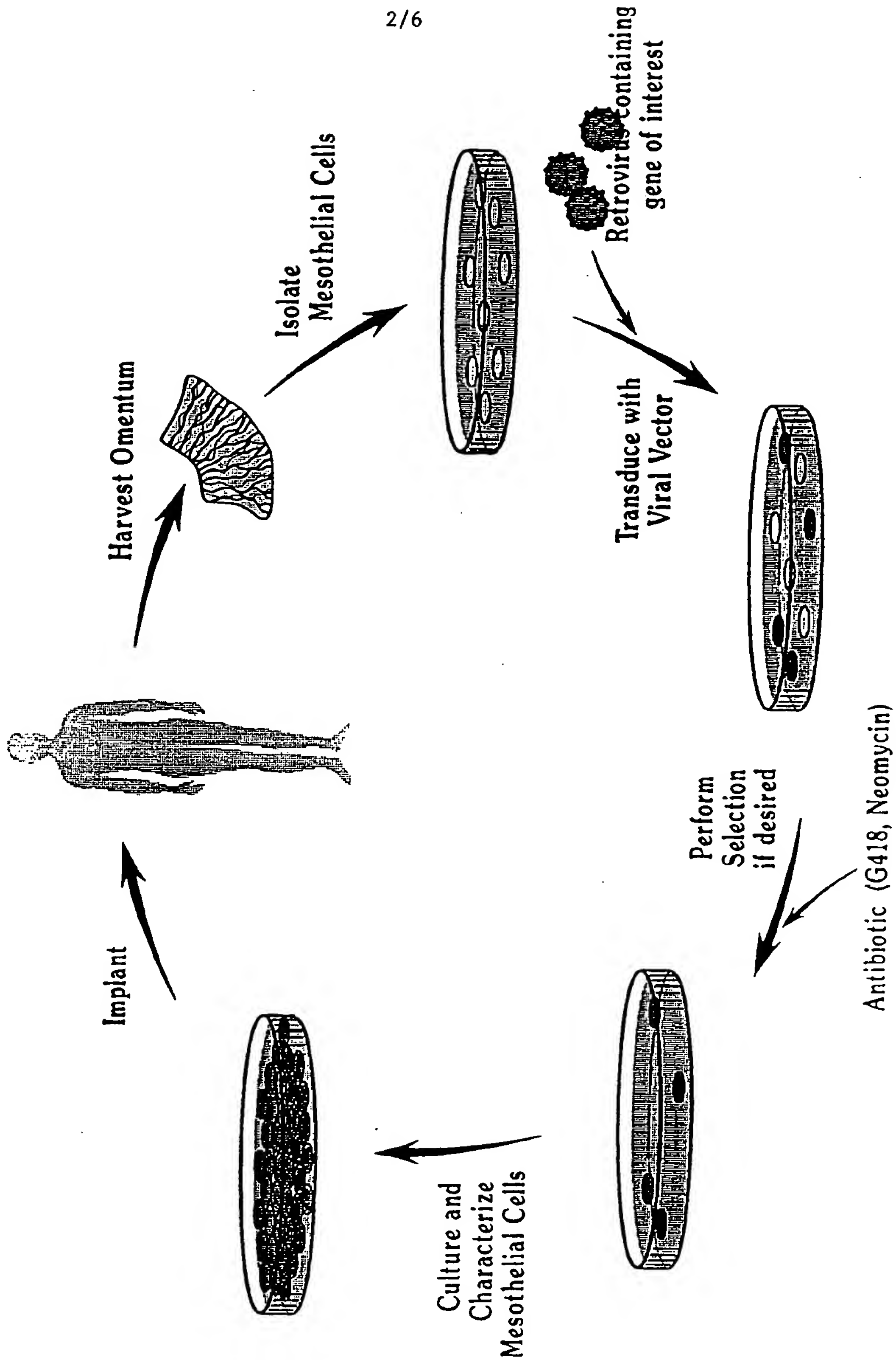

Harvest Omentum
Isolate
Mesothelial Cells
Retrovirus containing
gene of interest
Transduce with
Viral Vector
Perform
Selection
if desired
Antibiotic (G418, Neomycin)
Culture and
Characterize
Mesothelial Cells
Implant

Figure 2

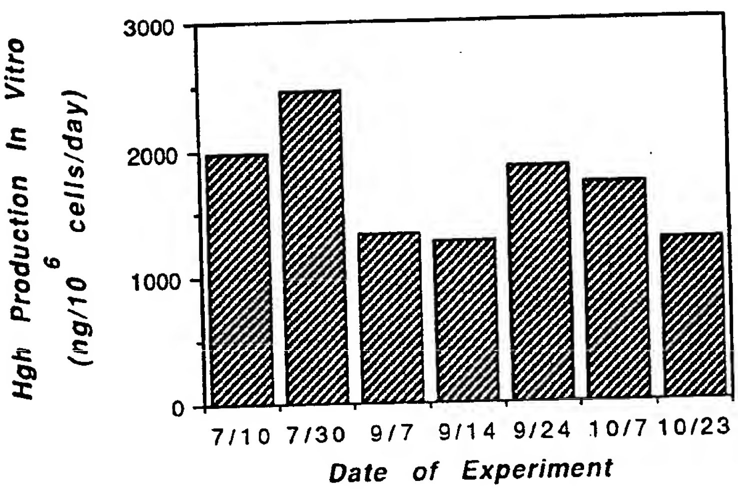

Hgh Production In Vitro
(ng/10^6 cells/day)
3000
2000
1000
0
7/10
7/30
9/7
9/14
9/24
10/7
10/23
Date of Experiment

Figure 3

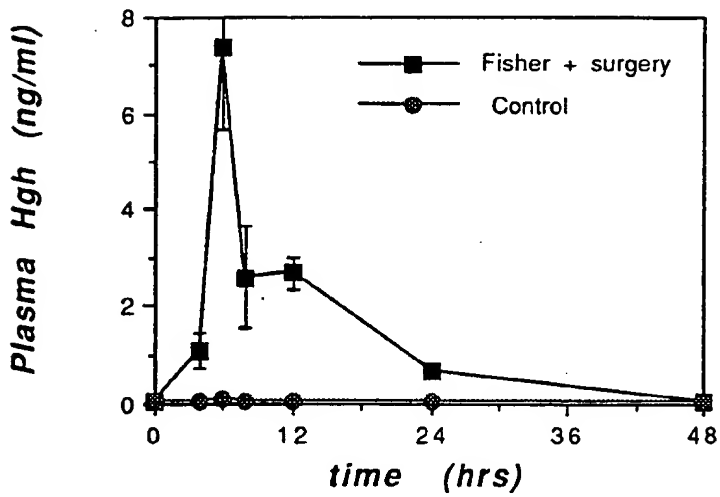


Figure 4A

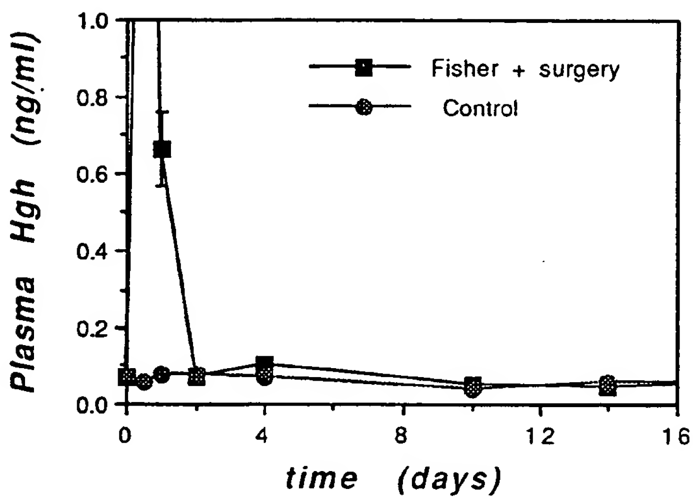


Figure 4B

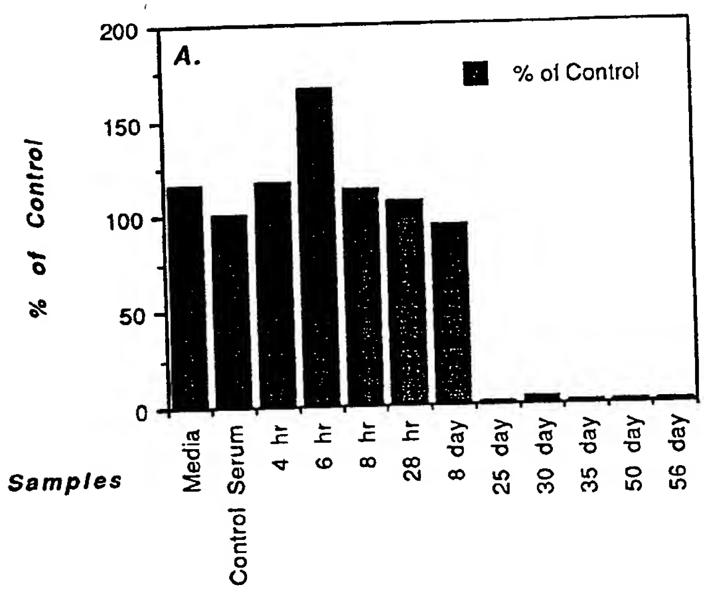


Figure 5A

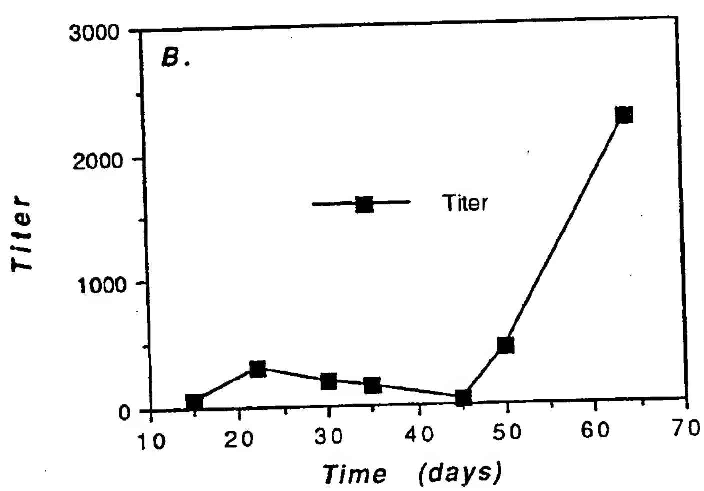


Figure 5B

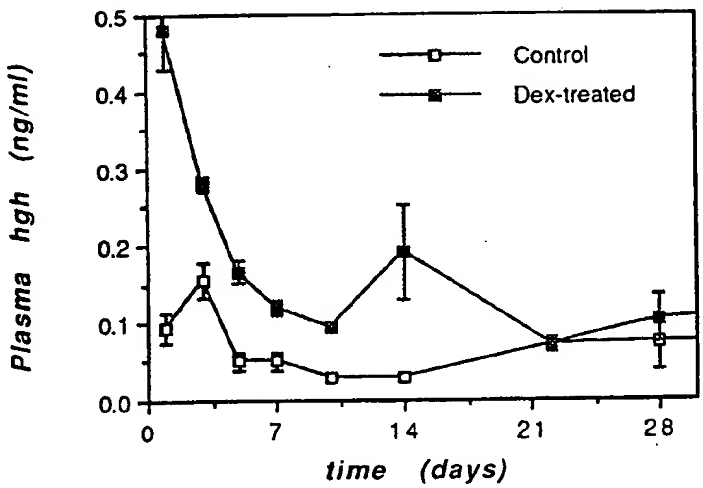


Figure 6

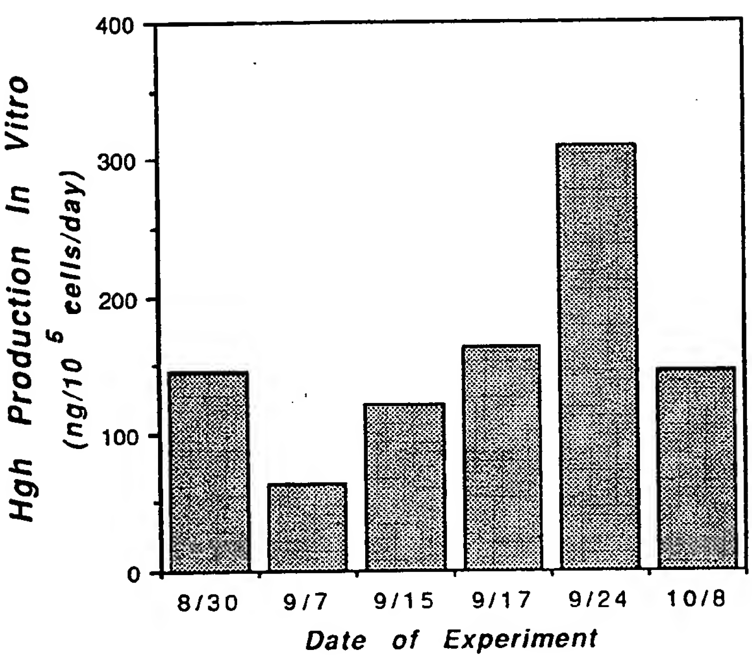


Figure 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/06809

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/85 C12N15/18 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF CELLULAR BIOCHEMISTRY vol. SUPPL, no. 18A , 4 January 1994 page 244 NAGY, J. A. ET AL. 'Mesothelial cell gene therapy' see abstract DZ 412 & Keystone Symposium on gene therapy Copper mountain, USA 15-22 january 1994 ---	1-44
Y	MEDECINE SCIENCES vol. 9, no. 2 , February 1993 pages 208 - 210 DANOS, O. ET AL. 'Réimplantation de cellules génétiquement modifiées dans des néo-organes vascularisés' see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 1994	- 4 -10- 1994
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/06809

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,89 03994 (US DEPT HEALTH & HUMAN US SEC OF COMMERCE) 5 May 1989 see the whole document ---	1
Y	DE,A,41 23 629 (BODZIONY, J.) 20 February 1992 see the whole document ---	1
Y	DE,A,40 11 100 (BODZIONY J.) 10 October 1991 see the whole document ---	1
Y	DE,A,40 01 319 (BODZIONY J.) 25 July 1991 see the whole document -----	1

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/06809

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : For claims 34-44, as far as they concern a method of treatment of the human/animal bodym the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.